IMPLANTATION IN NORMAL AND VITAMIN E DEFICIENT RATS[1]

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TWO FIGURES

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Whether vitamin E is essential for the capacity of the rat to become pregnant is a controversial question. In previous work, the influence of alpha-tocopherol acetate on the implantation rate of old rats had been studied (Kaunitz and Slanetz, '47). Experiments concerned with the influence of vitamin E deficiency upon implantation during the entire life of the rat are reported below.

METHODS

A highly inbred colony of albino rats was maintained on the vitamin E deficient diet given in table 1. The tocopherol content of this diet, estimated by an internal standard method (Kaunitz and Beaver, '44), was from 0.2 to 0.4 mg per 100 gm of diet, affording a daily intake of about 20–40 μg to an adult rat.[2] The controls received the same diet supplemented by 3 mg synthetic dl-alpha-tocopherol acetate per 100 gm diet, allowing a daily intake of approximately 300 μg.[3] Rats of the

[1] Aided by a grant from the John and Mary R. Markle Foundation.
[2] We are indebted to Drs. Philip L. Harris and Mary L. Quaife of the Distillation Products, Inc. for their help in the examination of lard samples.
[3] Dr. Leo Pirk of Homann-LaRoche, Inc., kindly supplied us with the synthetic dl-alpha-tocopherol acetate and the other vitamins used.

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1st to 8th generations, inclusive, raised on this diet were used. Some of the rats had received 1 or 2 doses of 1–3 mg of alpha-tocopherol acetate several months before they were used in the mating experiments recorded in figure 2.

Mating was carried out by leaving the female with the selected male for 5 days. On and subsequent to the 15th day after mating had begun, the animal was repeatedly inspected grossly for the placental sign,[4] and its weight was taken

TABLE 1

Composition of vitamin E deficient diet

BASAL MIXTURE		SUPPLEMENTS OF BASAL MIXTURE	
	%		*mg/kilo*
Casein, crude	30	Thiamine chloride	2
Cerelose	54	Riboflavin	4
Lard, commercial	10	Pyridoxine	4
Salt mixture (Hawk-Oser)[1]	4	Calcium pantothenate	10
Celluration	2	p-Amino benzoic acid	300
		Choline	1000
		Inositol	1000
		Vitamin K	4
		Oleum percomorphum	200

[1] Hawk, P. B., B. Oser and W. H. Summerson 1947 Practical Physiological Chemistry, 12th Edition. See page 1273. The Blakiston Co., Philadelphia.

daily. A positive placental sign accompanied by a gradual weight gain followed by weight loss was taken as proof of a resorption gestation. If the placental sign was persistently absent, and if the weight was constant, the absence of pregnancy was recorded.[5] The results obtained in this manner

[4] The "placental sign" refers to the appearance of blood in the rat's vagina 13–14 days after a mating. It is caused by the development of placental tissue.

[5] Some of the rats were used for matings after a previous resorption gestation or normal one had occurred, but at least 4 weeks were permitted to elapse after the resorption gestation or the weaning of the young. Contrary to the experiences of A. L. Bacharach and E. Allchorne (Biochemical Journal, 1938, *32:* 1298), we did not observe a drop in the implantation rate of animals that had previously had either a resorption or normal gestation, if animals of the same age were compared with virginal rats. This difference may be due to the fact that in Bacharach and Allchorne's experiments, the second mating was done 5–8 days after the termination of the first gestation.

were not always beyond question, particularly in rats below 2 and above 7 months of age. Therefore, laparatomies were carried out in more than 100 cases, and the uteri grossly examined for implantations. The answer disclosed by the laparatomy coincided in more than 90% of the cases with the previous "clinical diagnosis." We are therefore confident that the number of errors which may have occurred in the tests not checked by laparatomy was insignificant. Some

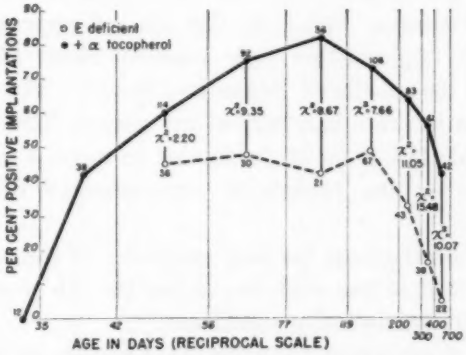

Fig. 1 Implantation rate in rats on a purified vitamin E deficient diet and on the same ration supplemented by 3 mg dl-alpha-tocopherol acetate per 100 gm diet. The numbers close to the circles indicate the number of experiments. Chi square denotes the probability factor.

pseudopregnancies may have been registered as resorption gestations, but the avoidance of this error would only have enhanced the significance of the differences.

The males used for the matings were derived from the same colony. They were raised on the deficient diet supplemented by 3 mg alpha-tocopherol acetate per 100 gm of diet. A negative mating test was accepted for the charts only if the male had proved to be fertile before and after the negative test; the weights were taken at intervals of 1 to 2 weeks and they were used only if no weight losses had occurred.

EXPERIMENTAL

In figure 1 are recorded the results of the mating experiments with rats on the vitamin E deficient diet and the same diet supplemented by alpha-tocopherol acetate. No implantations were observed among the animals on the diet supplemented by alpha-tocopherol acetate before the 35th day; thereafter, the rate of implantation went up steeply. This agrees with observations on the occurrence of the first estrus in rats (Engle et al., '37), and their attainment of sexual maturity (Blandau, '43). At the age of approximately 3 months, the implantation rate reached about 85%, which agrees with the results of Evans and Burr's ('27) studies on the implantation rate in a normal rat colony. Thereafter, the rate declined; but, even at the age of 18 months, nearly half of the rats on the tocopherol supplemented diet became pregnant.

In the deficient group, the implantation rate ran consistently lower than that of the controls. After the 9th week, the differences were statistically significant.[6]

In figure 2 are given the individual results of the mating tests and the body weights of the vitamin E deficient rats above the age of 150 days. One group had received 1 or 2 oral administrations of 1–3 mg alpha-tocopherol several months before the matings were recorded; the second group had been given no supplements. The administration of the tocopherol supplements several months before these experiments increased the body weights and the implantation rate. Implantations in the group without supplement were very rare after the 7th month, which is in good agreement with results of Emerson and Evans ('39).

[6] The chi square was calculated according to George W. Snedecor (Ames, Iowa, 1940). If chi square is greater than 3.8 the probability of an error is below 5%. We are indebted to Drs. Theodore F. Zucker and Lois Zucker for their help in the statistical analysis of the results.

DISCUSSION

Neither dietary deficiencies other than that of vitamin E nor infections can explain the low implantation rate in the vitamin E deficient rats. Dietary deficiencies other than of vitamin E can be ruled out because addition of alpha-tocopherol to the basic diet increased the implantation rate to normal. Infections of the genital organs may have occasionally

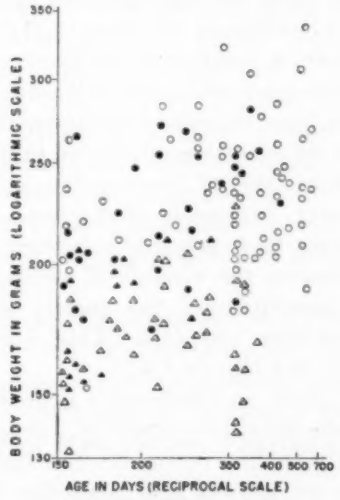

Fig. 2 Implantation rate in vitamin E deficient rats. The circles indicate rats which had received 1–3 oral supplements of 1–3 mg alpha-tocopherol acetate several months before the experiment was recorded. The triangles refer to rats without tocopherol supplements. Full circles or triangles denote pregnancy; hollow circles or triangles signify absence of implantation.

interfered with implantation in older animals; but they were not responsible for implantation failure in most cases because single tocopherol doses administered after mating increased the implantation rate significantly (Kaunitz and Slanetz, '47). Moreover, infections of the genital organs in deficient rats under 5 months of age are extremely rare. It seems, therefore, very probable that the effect of tocopherol

on the implantation rate is specific and not the result of secondary factors.[7]

Emerson and Evans ('39) as well as Goettsch and Pappenheimer ('41), found normal implantation rates in young rats on a vitamin E deficient diet. In the experiments of these authors, a diet was used which contained at least 20% lard and 8–12% dried yeast. These diets very probably permitted a daily intake of 40–80 μg of tocopherol for an adult rat in contrast to the 20–40 μg per day afforded by the diet used in our experiments. In view of the fact that a daily intake of 55 μg is, under certain conditions, on the border line of the requirements for normal gestations (Mason and Filer, '47), the tocopherol contents of some experimental diets may well have obscured the effect of tocopherol on implantation. Even with 20–40 μg, about 50% of the younger rats became pregnant. It is, however, extremely dangerous to compare tocopherol requirements in experiments carried on with widely differing diets because the vitamin E needs are greatly influenced by the quantity and the quality of many dietary constituents, especially the fats. This has recently been stressed by Mason and Harris ('47).

The beneficial effect on subsequent growth and implantation in female rats of single doses of 1–3 mg of tocopherol administered early in life resembles the influence of single tocopherol doses on growth and testicular development in males (Kaunitz, '46; Kaunitz et al., '44).

[7] We are indebted to Dr. Charles E. Tobin of the Department of Anatomy, University of Rochester, for this communication:

"An inbred strain of black mice obtained from Dr. Strong at Yale and known as the C-57 strain were started on a vitamin E-deficient diet (diet 69) on the day of delivery. If the females from these litters are mated with normal males between 75 and 100 days of age they will become pregnant and resorb their young. This fetal resorption can be prevented by oral administration of tocopherols or implantation of various tocopherol esters in the form of pellets subcutaneously. However, if such E-deficient females are kept on the E-deficient diet for 100 to 150 days or more before mating with normal males, pregnancy will not ensue although copulation plugs or sperm will be found in the vaginae of these animals for several successive estrous cycles. I have observed this phenomenon in six mice which were studied especially for this purpose although it has occurred in other animals previously, and is being found in more animals now under observation."

Storage and gradual utilization of the single dose of 1–3 mg of tocopherol can hardly explain the effects lasting for at least 1 year because this would have provided only 3–10 μg per day, which amount is within the limits of error of dietary intake. A valid explanation for this effect has not as yet been given. The protracted effect of single doses of tocopherol administered early in life makes more difficult the calculation of "daily" requirments.

In figure 2, it can be seen that no mating experiments were carried out on rats older than 1 year in the group without tocopherol supplements. Ten experiments on "single dose" animals over 500 days old were recorded. This was possible because the life span of a rat given a single supplement before the 5th month is considerably longer than that of an animal without such a supplement. Whether or not this is a specific tocopherol effect is not clear; it may be indicative of a higher resistance to respiratory and genital infections in the protected group.

The mechanism of the implantation failure in vitamin E deficiency is not known. Experiments dealing with this question are being carried out in cooperation with Dr. Richard J. Blandau of the Department of Anatomy, The University of Rochester School of Medicine and Dentistry.

SUMMARY

1. The implantation rate in a highly inbred rat colony maintained on a complete, purified diet was determined until the rats were 2½ years old.

2. The implantation rate of rats maintained on the same diet without the tocopherol supplement was found to be significantly lower than that of the control group after the rats were 9 weeks old. Tocopherol is essential for the ability of the rat to become pregnant.

3. Single doses of 1–3 mg of alpha-tocopherol administered early in life have a beneficial effect on subsequent growth, implantation rate, and life span of the female rat.

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CHOLINE DEFICIENCY IN THE BABY PIG

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TEN FIGURES

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In previous papers it has been shown that day-old pigs can be raised to weaning age on a completely "synthetic" diet made up to simulate cow's milk (Johnson, James and Krider, '47, '48). With such a synthetic diet, it is possible to omit or alter 1 or more constitutents at a time, thus making possible investigation of the nutritional requirements of the baby pig. Such information should be useful in studies on human infant nutrition.

In this paper we wish to report the production of a choline deficiency in baby pigs by omitting choline from the synthetic milk ration.

Wintrobe, Miller, Follis, Stein, Mushatt and Humphreys ('42) fed 3- to 4-month-old pigs a choline-deficient diet and observed an increased amount of fat in the livers and some abnormality of gait, but found no effect on growth. Ellis, Madsen, and Miller ('43) reported that choline is necessary in addition to pantothenic acid and pyridoxine for protection from locomotor incoordination resulting from nerve degeneration, but that growth is not affected. Ensminger, Bowland, and Cunha ('47) fed sows a choline-low ration and found that most of the baby pigs from these sows failed to survive.

EXPERIMENTAL

Nine pigs, 6 1-day-old Chester White pigs from 1 litter and 3 4-day-old Duroc-Jersey pigs from another litter, were

divided into 3 groups consisting of 1 Duroc-Jersey and 2 Chester White pigs in each group. Group 1 was fed the "complete synthetic milk" ration given in table 1. Group 2 was fed the same ration with the choline, inositol and *p*-aminobenzoic acid omitted; and group 3, the complete ration with

TABLE 1

Composition of "synthetic milk" ration

	%
Casein (Labco, vitamin-free)	30
Glucose (cerelose)	37.4
Mineral salts [1]	6
Lard	26.6

Made up and homogenized into a milk containing 13% solids including 4% lard (liquid basis).

The following vitamins [2] were added to the complete diet per 1000 gm of milk:

Thiamine	0.65 mg	*p*-Aminobenzoic acid	2.6 mg
Riboflavin	1.30 mg	Pteroylglutamic acid	0.052 mg
Pyridoxine	1.30 mg	Biotin	0.01 mg
Calcium pantothenate	7.8 mg	α-Tocopherol acetate	1.0 mg
Inositol	26.0 mg	2 methyl-1,4-naphthoquinone	0.26 mg
Choline	260.0 mg	Vitamin A	1000 I.U.
Nicotinic acid	2.6 mg	Vitamin D_2	100 I.U.

[1] See Johnson et al. ('48).

[2] The thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, biotin and α-tocopherol acetate used in this experiment were very generously supplied by Hoffmann-La Roche, Inc., Nutley, New Jersey, through the courtesy of Dr. J. C. Bauernfeind.

Pteroylglutamic acid was generously supplied by the Lederle Laboratories Division, American Cyanamid Co., Pearl River, New York.

Inositol was generously supplied by the A. E. Staley Manufacturing Co., Decatur, Illinois.

choline omitted. All pigs were fed sulfathalidine [1] at 2% on the dry basis to inhibit intestinal synthesis. The pigs were housed and fed ad libitum as previously reported (Johnson et al., '48).

The lard used in this ration was analyzed for choline by the Neurospora procedure of Horowitz and Beadle ('43) and

[1] Sulfathalidine (phthalylsulfathiazole) was generously supplied by Sharpe and Dohme, Philadelphia, Pennsylvania, through the courtesy of Dr. S. F. Scheidy.

was found to contain 10.6 γ of choline per gm, which equals 2.4 γ/ml for the "synthetic milk" ration. One sample of sow's milk was found to contain 273 γ/ml. Engel ('43) gives 147 γ/ml for the choline content of cows' milk.

The smallest of the Chester-White pigs weighed only 900 gm when started on experiment and died on the 2nd day, leaving only 2 pigs (1 Chester-White and 1 Duroc-Jersey) in the positive control group. The rest of the pigs were kept on the experiment for 8 weeks without further loss. During this experimental period blood samples were taken 4 times (at 2-week intervals), and hemoglobin concentration and red blood cell and white cell counts were determined.

At the end of the 8-week experimental period liver biopsies were taken of all animals.[2] One small piece from each liver was frozen immediately, sectioned with the freezing microtome, and stained with Sudan IV and light green.[3] Another small piece was fixed, sectioned by the paraffin method, and stained with Harris's hematoxylin and Orange G.

RESULTS AND DISCUSSION

The growth curves of the experimental pigs are plotted in chart 1. Due to the marked difference in weights of the Duroc-Jersey as compared to the Chester-White pigs, the growth data are plotted as per cent of initial weight. Since there was no difference ($P = 0.79$) in percentage gains of the pigs in group 2 and group 3 (choline-, *p*-aminobenzoic acid-, inositol-low vs. choline-low), they are treated as 1 choline-deficient group, and the 2 curves given are the averages of the percentage gains of the control and of the choline-deficient groups. The points plotted, however, are the individual data for each animal. In terms of actual weights, the Duroc-Jersey pig in the positive control group reached 24.55 kg (54 lbs.) at 8 weeks (i.e., good growth).

[2] We wish to express our appreciation to Dr. L. E. Boley and Dr. H. Hardenbrook for performing the operations.

[3] We wish to express our appreciation to Prof. F. B. Adamstone of the Department of Zoology for sectioning and preparation of the slides from the frozen material.

Statistical treatment of the growth data gives the following probabilities: control group vs. group 2 (minus choline, inositol, *p.*-aminobenzoic acid) P = 0.03 (Fisher, '44); control group vs. group 3 (minus choline) P = 0.05; control group vs. groups 2 and 3 (both groups minus choline) P = <0.01; while for group 2 vs. group 3, P = 0.79. That is, the omission of choline from the "synthetic milk" ration of these baby

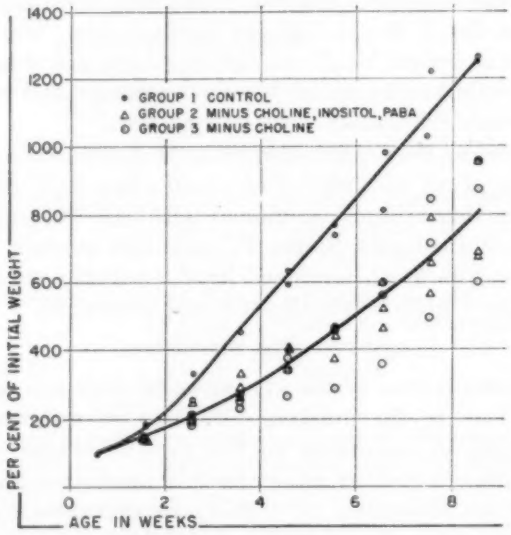

Chart 1 Growth curves of baby pigs on "complete" vs. choline-low "synthetic milk" diet.

pigs markedly retarded their growth. Plate 1 shows the pictures of pigs 2 and 3 (positive controls, group 1) and 7, 8, and 9 (minus choline, group 3) at 5 weeks. The differences in size of these pairs, which initially weighed approximately the same, are quite evident. As well as difference in size, the choline-deficient pigs presented in some cases a generally unthrifty appearance and rough haircoat.

The hemoglobin values and red blood cell counts are given in table 2. No significant differences between groups with

respect to hemoglobin concentration or white cell counts were found. However, the increases in red blood cell count during the 8-week experimental period did show significant differences. The control group showed an average increase of 2.37 million cells per mm^3, while the choline low groups showed an average increase of only 0.86 million cells per mm^3 ($P = 0.044$).

TABLE 2

Red blood cell counts and hemoglobin concentrations for baby pigs fed the "synthetic milk" rations

GROUP	WEEKS ON EXPERIMENT			
	2	4	6	8
	Average red blood cell counts in millions/mm^3			
1 (control)	6.64	7.78	8.22	9.02
2 (minus choline, inositol and *p*-aminobenzoic acid)	6.72	6.39	7.63	7.61
3 (minus choline)	6.54	6.90	7.45	7.37
	Average hemoglobin concentration in gm/100 ml			
1	13.3	13.8	11.8	13.2
2	11.5	10.5	12.3	12.4
3	10.9	10.2	11.4	11.8

The liver sections taken by biopsy were examined histologically, and plate 2 illustrates the results found in each group of baby pigs. As can be seen, there was severe fatty infiltration in the livers of the pigs from both groups 2 and 3, while the livers from the control group were normal in appearance. There appears to be somewhat more fat deposition in the livers of the pigs in group 2 (minus choline, inositol and *p*-aminobenzoic acid, plate 2, figs. 6 and 9) than in the livers from group 3 (minus choline, figs. 5 and 8), which may indicate some role of inositol in fatty liver formation in the baby pig. However, this must be further investigated on a greater number of pigs before a definite conclusion can be reached.

SUMMARY

One and 4-day-old pigs have been raised on a "synthetic" diet made up to simulate milk.

On this diet, which contains 30% protein as casein (dry basis), the baby pig has been found to require choline. When choline was omitted from the diet, the pigs gained weight at a slower rate and developed fatty infiltration of the liver. The pigs were fed ad libitum. The choline-deficient pigs did not show as good erythrocyte formation, as determined by red blood cell count increases during the 8 weeks to weaning, as did baby pigs receiving the "complete synthetic milk" diet. Definite evidence was not obtained for a requirement of inositol or *p*-aminobenzoic acid when their combined deficiencies were superimposed on a choline deficiency, although their omission from the diet appeared to accentuate the degree of fatty infiltration of the livers.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

Photographs of pigs after 5 weeks on experiment

1 Shows pig 2 on the "complete" diet and pig 7 on the choline-low diet.
2 Shows pig 2 on the "complete" diet and pig 8 on the choline-low diet.
3 Shows pig 3 on the "complete' 'diet and pig 9 on the choline-low diet.

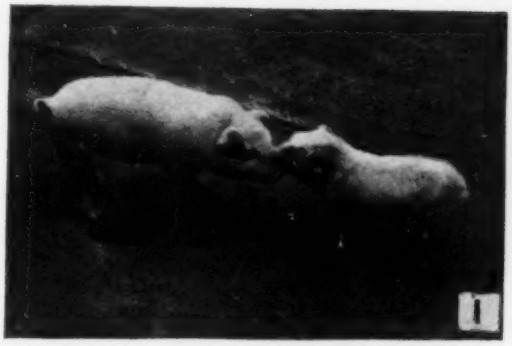
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PLATE 2

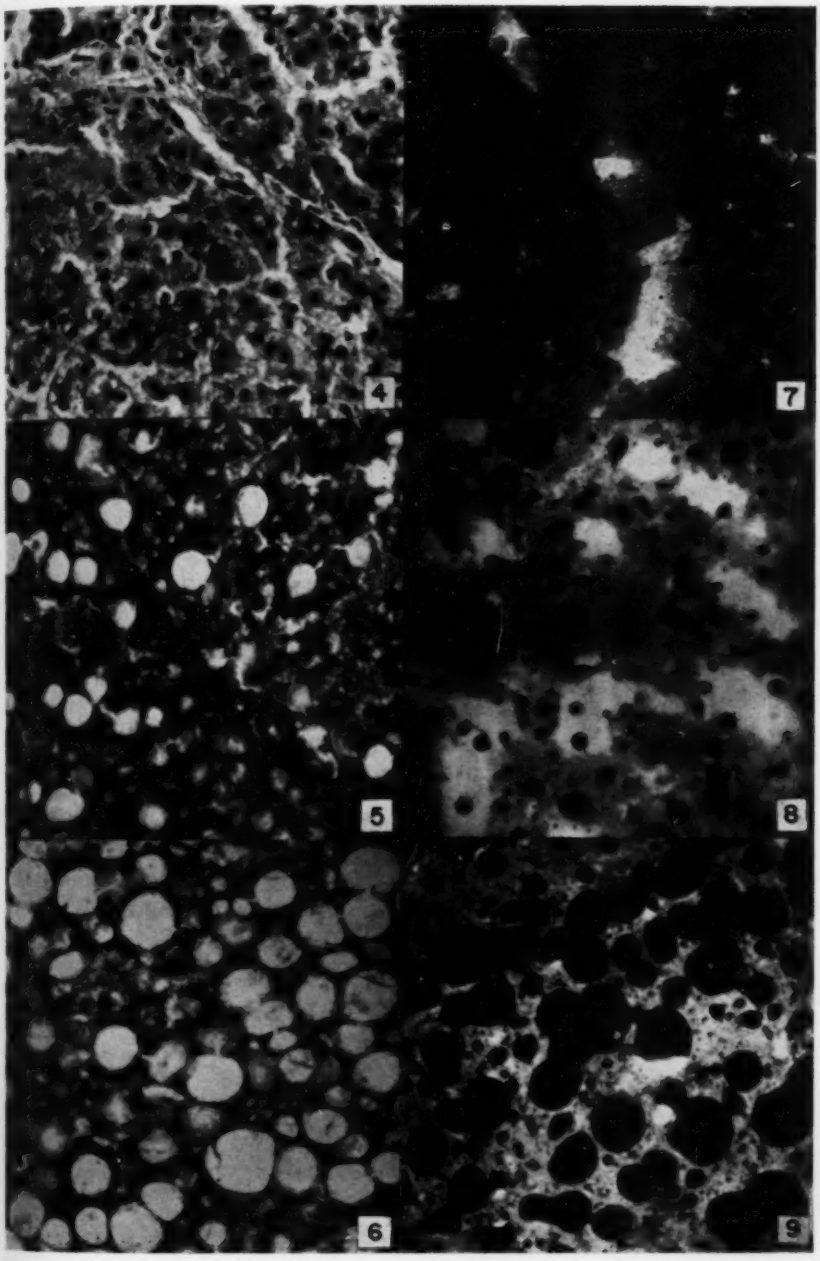
EXPLANATION OF FIGURES

Photomicrographs of liver sections of pigs at termination of experiment (8 weeks). Magnification × 225 (original × 300 reduced ¼). 4, 5, and 6 are paraffin sections stained with Harris's hematoxylin and Orange G, 7, 8, and 9 are frozen sections stained with Sudan IV and light green. Tissues in figures 7 and 8 were left frozen for some time before sectioning and hence some deterioration of tissue is shown by light spaces.

4 and 7 Liver from group 1 control (pig 2). Shows normal appearance and normal distribution of fat.

5 and 8 Liver from group 3, choline omitted (pig 8). Some fat infiltrations. Figure 5 shows fat removed in process of making paraffin section. Fat droplets are stained with Sudan IV in figure 8.

6 and 9 Liver from group 2, choline, inositol, and *p*-aminobenzoic acid omitted (pig 4). Note the large amount of fat infiltration.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POTASSIUM REQUIREMENT OF THE CHICK[1]

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ONE FIGURE

(Received for publication April 1, 1948)

It has long been known that potassium plays a vital role in animal physiology. In spite of this, knowledge concerning the quantitative requirements of animals for this element is still in an unsatisfactory state. To a lesser extent this is also true of the deficiency symptoms which appear when suboptimum amounts of potassium are fed. Most of the work in this connection has been done with the rat and considerable variability in results has been reported. The earlier work with this species has been clarified to some extent by the recent report of Kornberg and Endicott ('46) who described the following symptoms in rats fed diets very low in potassium: prompt failure of growth, edema, multiple lesions in many tissues, and death. A dietary potassium level of 0.17% was found to be adequate for growth and the prevention of lesions.

While studying potassium deficiency in dogs, Ruegamer, Elvehjem and Hart ('46) observed symptoms which included cessation of growth, "stiff neck" and paralysis of the limbs, discolored teeth and hemoconcentration. Hughes and Ittner ('42) have reported that young pigs require approximately 0.15% of potassium in the diet for optimum growth. Apparently the only report of potassium deficiency in the chick is that of Ben Dor ('41). He observed retarded growth and

[1] This work was supported in part by a grant from the International Minerals and Chemical Corporation, Chicago, Illinois.

high mortality in chicks fed a low-potassium diet and concluded that at least 0.17% of potassium in the diet was necessary for maximum growth.

The potassium requirement of chicks has been reinvestigated in this laboratory in connection with a general study of factors affecting mineral metabolism in this species. Such a study was prompted by the limited information available and the need for a better characterization of the symptoms of potassium deficiency. Furthermore, unpublished results had been obtained which suggested a relationship between the potassium and phosphorus requirements of chicks. This possibility was also given further attention in the experiment reported here.

EXPERIMENTAL PROCEDURE

In this experiment it was desired to study both the potassium requirement and the possible relationship between this requirement and the amount of phosphorus present in the diet. Accordingly, the 7 different levels of potassium shown in table 1 were each fed to lots of chicks receiving 0.4% and 0.6% phosphorus, respectively. This smaller amount, 0.4%, is near the generally accepted minimum requirement for chicks while 0.6% is more nearly representative of an optimum allowance of this element. The 2 groups of chicks served not only to test the relationship between phosphorus and potassium requirements, but also as a double check on the quantitative requirement for potassium.

Except with respect to potassium, the basal diet used in this experiment was adequate in all nutrients known to be required by the chick. The diet of the lots receiving 0.4% phosphorus had the following percentage composition: cornstarch 61.55, blood fibrin 25, gelatin 5, cellophane 2, liver "L" 1, soybean oil 2, dicalcium phosphate 1.5, salts 1.45[2] and

[2] Salts (per cent of diet): $CaCO_3$, 0.75; NaCl, 0.60; $MgSO_4 \cdot 7H_2O$, 0.06; $MnSO_4 \cdot 4H_2O$, 0.03; $ZnCl_2$, 0.001; $CoCl_2 \cdot 6H_2O$, 0.0003; NaI, 0.003; $CuSO_4 \cdot 5H_2O$, 0.0017.

vitamins 0.5.[3] The diet of the lots receiving 0.6% phosphorus was identical except that the dicalcium phosphate was increased sufficiently to raise the phosphorus content to 0.6% and the cornstarch was decreased accordingly. A calcium to phosphorus ratio of 1.9:1 was maintained in both instances by the addition of calcium carbonate. The basal diet contained 0.04% potassium as determined by analysis with the flame photometer.[4] The desired potassium levels were obtained by adding a solution of analytical grade KCl to the basal mixtures.

White Leghorn male chicks were used as experimental animals. Lots of 15 1-day old chicks were placed in electrically heated battery brooders and supplied the experimental diets ad libitum. During the 4 weeks' experimental period the chicks were observed for symptoms of potassium deficiency and post mortem examinations were made of the chicks which died. Individual weights were taken at weekly intervals. At the end of 4 weeks all surviving chicks were sacrificed, further observations were made on the deficient lots, and standard bone ash determinations were carried out on representative chicks from each lot.

RESULTS AND DISCUSSION

The results obtained with respect to growth, bone ash, and mortality are presented in table 1. The striking effect of potassium on growth, calcification and viability of chicks is evident from these data. In the case of the chicks receiving the marginal level of phosphorus, growth was increased progressively by additional potassium up to a level of 0.24% of the diet. For the chicks receiving the higher level of phosphorus growth was similarly increased by additions of potassium up to a level of 0.20%.

[3] Vitamins (mg per 100 gm): thiamine 0.3; riboflavin 0.5; calcium pantothenate 1.1; pyridoxine 0.5; niacin 1.75; vitamin K 0.22; folic acid 0.33; biotin 0.026; vitamin A alcohol 0.11; PABA 11.0; inositol 111; choline 222; tocopherols in oil 15; vitamin D_3 50 AOAC chick units.

[4] Dr. C. J. Barton made the analyses.

Calcification, as measured by the percentage of ash in fat-free, dry bone, was likewise benefited by supplementary potassium in the diet. In the case of the low phosphorus group, 0.20% potassium gave optimum bone ash values, and for the group receiving the higher phosphorus diet 0.16% potassium was adequate for optimum calcification. According to Cook and Robertson ('40), tibiae from day-old chicks contain approximately 36% ash. The percentage of bone ash found in chicks which received 0.08% potassium and 0.4% phosphorus represents a considerably lowered ratio of mineral matter to soft tissue deposited during growth. The poor

TABLE 1

The effect of different levels of potassium on growth, calcification, and mortality in chicks

LEVEL OF POTASSIUM	GROUP 1 (*0.4% Phosphorus*)			GROUP 2 (*0.6% Phosphorus*)		
	Wt. at 4 wks.	Bone ash	Mortality	Wt. at 4 wks.	Bone ash	Mortality
%	gm	%	%	gm	%	%
0.04	..	...	100	.....	...	100
0.08	85	23.6	87	147	41.3	87
0.12	185	38.3	33	221	43.0	13
0.16	298	41.6	0	322	47.8	7
0.20	311	43.4	0	368	46.7	0
0.24	332	42.8	0	344	46.7	7
0.50	334	43.9	0	357	47.6	0

calcification found in the low potassium lots, therefore, cannot be accounted for on the basis of retardation of growth alone.

The sparing action of phosphorus on the potassium requirement as measured by both growth and calcification points to an interrelationship in the metabolism of these 2 elements. In view of the fact that growth and calcification were better in the lots receiving 0.6% phosphorus than in those receiving 0.4%, the latter level of dietary phosphorus is probably too low for the rapidly growing chick.

These results indicate that the potassium requirement for maximum growth of the chick is 0.20–0.24% which is

somewhat higher than the 0.17% previously reported by Ben Dor ('41). This difference is probably due to different experimental conditions. The conclusions drawn from the earlier work were based on growth response to a diet which permitted only relatively slow growth even when supplemented with potassium. It is probable that the much faster rate of growth obtained in the experiment reported in this paper increased the potassium requirement. The fact that some chicks used by Ben Dor ('41) were maintained on an adequate commercial diet for a period before receiving experimental treatment may also have contributed to the difference in results obtained.

Continuous observations were made of the behavior and deficiency symptoms of the chicks in this experiment. Retarded growth and development were the most immediate symptoms of an inadequate potassium intake. The growth of all chicks receiving suboptimum amounts of potassium was retarded throughout the experiment. Chicks receiving the basal diets made virtually no gains in weight even though some of them survived for as long as 24 days. Mortality began by the 5th day in lots receiving the lower levels of potassium. The general inferiority of these lots was clearly evident by the end of the 1st week, and became progressively more marked (fig. 1). In potassium deficient lots many chicks gradually lost the use of their legs and were unable to stand or walk. Pasting around the vent and the excretion of excessive amounts of urates were common in deficient lots. Feed consumption by deficient chicks was reduced, but even those in the basal lots maintained an active interest in food.

Death in deficient chicks was generally preceded by difficulty in breathing, sometimes for several hours, and the heart rate, normally very high in the chick, was markedly slower. Tonic spasms preceding death were also common. During these spasms the legs were rigidly extended and frequently the head was retracted. The leg and other body muscles were sometimes seized with tremors. Rigidly extended legs at time of death were very characteristic. This inability of the

muscles to relax is not surprising in view of the known function of K^+ ions in reducing muscular contractility and favoring relaxation in contrast to Ca^{++} ions which favor contraction.

Characteristic post mortem findings included emaciation, enlarged gall bladder, and swollen kidneys with deposits of urates in the ureters and sometimes throughout the kidneys. In some cases the kidneys were twice the normal size. The intestines and ceca were frequently distended by material collected in them. Less common findings included hydropericardium, discoloring lesions on the surface of the liver, and thin, eroded areas in the intestinal walls.

Fig. 1 Comparison of potassium deficient and normal chicks at 2 weeks of age. The chick on the left received 0.04% potassium while the one on the right received 0.20% potassium.

SUMMARY

1. Rapidly growing, White Leghorn male chicks were found to require from 0.20–0.24% dietary potassium for maximum growth. The requirement for potassium was slightly higher when the phosphorus in the diet was at a marginal level than when an optimum amount of phosphorus was present; 0.16% potassium was adequate for the prevention of mortality.

2. Chicks receiving a marginal level of phosphorus in the diet required approximately 0.20% potassium for optimum calcification of the bones, while the corresponding require-

ment in the case of chicks receiving an optimum allowance of phosphorus was 0.16% potassium.

3. Symptoms of potassium deficiency in the chick included retarded growth, weakness, loss of use of legs and the excretion of large amounts of urates. Death was preceded or accompanied by tetanic seizures in which the muscles were unable to relax. Mortality commenced as early as the 5th day in deficient chicks, and no chicks receiving the basal diet, containing 0.04% potassium, survived the 4 weeks' experimental period. Post mortem examination revealed lesions in several organs, particularly the kidneys and ureters which were enlarged and usually congested with urates.

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AVAILABILITY OF AMINO ACIDS IN SOME FOODS[1]

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The nutritive value of a protein is dependent not only upon its content of essential amino acids (Rose, '38) but the biological availability of these amino acids as well. Incomplete digestion and absorption of an essential amino acid has the result of altering the effective composition of a food protein. The ultimate synthesis of body protein from the amino acids made available through digestion and absorption is dependent on a third factor, namely, the simultaneous appearance in the blood stream of each of the essential amino acids in suitable proportions. The reports of Elman ('39), Melnick, Oser and Weiss ('46), and Geiger ('47) emphasize that protein synthesis does not occur unless a complete mixture of the essential amino acids is present at one time.

Only limited data are to be found in the literature concerning the availability of amino acids in foodstuffs. That the availability of the individual amino acids might vary would be inferred from *in vitro* digestion studies (Mitchell and Hamilton, '29; Jones and Gersdorff, '33; Melnick, Oser and Weiss, '46) which indicate that amino acids are liberated from proteins at different rates characteristic of the amino acid or its

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linkage in the protein. In addition, it has been demonstrated (Chase and Lewis, '34) that specific amino acids are absorbed into the blood at different rates.

On the basis of sulfur balance studies with chicks, Evans and McGinnis ('47) concluded that the availability of cystine and methionine in soybean oil meal can be modified by the method of processing the meal. Methionine balance studies reported by Melnick et al. ('46) showed that 49% of the methionine in a sample of soybean oil meal appeared in the feces and hence was not available to the rat.

In the present investigation, the availability of all 10 of the essential amino acids in several foodstuffs was determined. Calculations were based on determination of the amino acids in the food and in the feces. Corrections for the metabolic amino acids in the feces were calculated from control periods in which a low protein egg ration was fed.

EXPERIMENTAL

The experimental techniques used in conducting the feeding tests were largely adaptations of the methods used by Mitchell and coworkers ('24, '26) in their investigations on the digestion and metabolism of proteins in foodstuffs. Groups of 5 rats each, weighing initially from 55 to 60 gm, were fed for a 24-hour period a stock ration containing 2% Fe_2O_3 as a feces marker. They were then fed ad libitum for 7 days an experimental ration which contained about 10% protein from a single source. Following this feeding they were again fed for 24 hours the stock ration containing the feces marker. All definitely red feces were discarded. Filter paper was placed in the bottom of the cages to absorb urine; an attempt was made to separate hair from the feces.

In order to determine the amount of the amino acids which appeared in the feces as a result of metabolic processes, such as the excretion of enzymes into the digestive tract, the experimental period was followed by a standardizing period of 7 days in which a low nitrogen ration was fed. This ration contained 6% defatted whole egg to give a protein content of

approximately 4%. It has been shown by Mitchell and Carman ('26) and Bricker and Mitchell ('47) that whole egg protein when fed in these amounts is completely digested and utilized. The composition of all diets is given in table 1.

Food composition was measured and feces collections were made for the experimental and standardizing periods. In order to control scattering of food, the rations were fed in the

TABLE 1

Composition of rations

CONSTITUENTS	ROAST BEEF RATION	COTTONSEED FLOUR RATION	PEANUT FLOUR RATION	WHEAT RATION	EGG RATION
	%	%	%	%	%
Roast beef [1]	13.8	..	..	..	..
Cottonseed flour [1] (Proflo)	..	18.5	..	..	..
Peanut flour [1]	..	..	16.4	..	..
Wheat [1]	..	..	..	64.5	..
Defatted whole egg [1]	..	..	..	..	6
Sucrose	10	10	10	10	10
Vitaminized starch [2]	5	5	5	5	5
Starch	52.2	47.5	49.6	1.5	60
Wood pulp (alpha-flock B) [3]	2	2	2	2	2
Butter fat	8	8	8	8	8
Cod liver oil	2	2	2	2	2
Salt mixture [4]	4	4	4	4	4
NaCl	1	1	1	1	1
$BaSO_4$	2	2	2	2	2

[1] Crude protein content of materials (N × 6.25): Roast beef, 72.25; cottonseed flour, 54.07; peanut flour, 61.00; wheat, 15.54; egg, 69.88.

[2] Mitchell et al. ('45).

[3] Brown Co., Berlin, New Hampshire.

[4] Wesson ('32) NaF omitted.

form of a paste containing 10 ml of a dilute liver extract, prepared as indicated below, and 15 gm of the dry ration. The use of the liver extract was an important factor in maintaining satisfactory food consumption. Food residues were dried and weighed. Moisture determinations were made in order to adjust all food residue weights to the original moisture content of the prepared rations.

Preparation of liver extract

Five pounds of fresh beef liver were ground and then homogenized in the Waring Blendor with 6 liters of methanol. The protein was removed by centrifugation. The methanol was removed by distillation at reduced pressure and the residue dissolved in 5 liters of water and filtered. The nitrogen content was 0.58 mg per ml; the amino acid content was considered insignificant.

Preparation of roast beef

The roast beef sample was well done. Three pounds of boned prime rib were roasted for 2¼ hours at 350°F. The lean meat was ground in a food chopper and dehydrated at room temperature with the apparatus described by Lyman et al. ('46a). In order to facilitate thorough mixing of the ration, the meat was reground after drying.

Microbiological methods were used for the determination of the essential amino acids in the food and feces. Valine, leucine, and isoleucine were determined with *L. arabinosus* 17–5 (Kuiken et al., '43). *Streptococcus faecalis* (American Type Culture Collection no. 8043) was used for threonine, histidine (Lyman et al., '47) and tryptophane assays (Kuiken et al., '47), while *L. mesenteroides* P-60 was used to determine methionine (Lyman et al., '46b), arginine, lysine, and phenylalanine. The amino acid composition of the materials studied is given in table 2.

RESULTS AND DISCUSSION

Illustrative data for a single amino acid, lysine in roast beef, are given in table 3. The data show that the endogenous amino acid excretion was roughly proportional to the amount of food eaten. This is in accordance with the observations of Mitchell ('24) and Schneider ('35) on endogenous nitrogen excretion. The calculation of true amino acid availabilities was based on this concept. In general the correction to be applied to apparent availabilities was quite small.

TABLE 2

Amino acid composition of materials studied
(Expressed as percentage of the crude protein, N × 6.25)

AMINO ACID	ROAST BEEF	COTTONSEED FLOUR	PEANUT FLOUR	WHEAT
Arginine	6.53	11.30	11.26	4.38
Histidine	3.27	2.57	2.16	2.51
Isoleucine	4.43	3.51	3.85	3.03
Leucine	8.77	6.23	6.74	6.82
Lysine	9.98	4.16	3.20	2.90
Methionine	3.13	1.61	1.02	1.35
Phenylalanine	4.25	5.20	5.08	5.15
Threonine	4.90	3.36	2.67	2.83
Tryptophane	1.30	1.46	1.20	1.35
Valine	6.03	4.84	4.64	4.50

TABLE 3

Metabolic data and the calculation of lysine availability in roast beef

RAT NUMBER	1	2	3	4	5
Metabolic data: roast beef ration, 9.97% protein					
Food intake (gm)	71.7	74.8	56.9	69.1	88.2
Lysine in food eaten (gm)	0.713	0.744	0.566	0.688	0.877
Weight of feces (gm)	4.487	4.432	3.506	4.877	5.452
Lysine in feces (%)	0.64	0.66	0.83	0.78	0.64
Total lysine in feces (mg)	28.7	29.2	29.1	38.0	34.9
Metabolic data: egg ration, 4.19% protein					
Food intake (gm)	62.6	65.0	45.3	46.0	69.2
Weight of feces (gm)	3.908	4.283	2.772	3.017	4.610
Lysine in feces (%)	0.66	0.66	0.66	0.66	0.63
Total lysine in feces (mg)	26	28	18	20	29
Lysine in feces per gram food eaten (mg)	0.42	0.43	0.40	0.43	0.42
Derived data					
Fecal lysine of metabolic origin (mg)	30.1	32.1	22.8	29.7	37.0
Fecal lysine of dietary origin (mg)	—1.4	—2.9	6.3	8.3	—2.1
Apparent lysine availability (%)	95.9	96.1	94.9	94.5	96.0
True lysine availability (%)	100.1	100.4	98.9	98.8	100.2

The essential amino acids in roast beef were found to be completely available to the rat under the described experimental conditions. As is shown in table 4 the true availabilities of these amino acids ranged from 99.2 to 100.7%. The total nitrogen of the meat protein was also found to be completely available. This result was to be expected in view of the finding of Mitchell et al. ('36) that the protein of beef round is completely digestible. It is of interest that roasting the beef did not decrease the availability of the amino acids.

TABLE 4

True availability of amino acids in roast beef

AMINO ACID	EXPERIMENT 1						EXPERIMENT 2
	Rat number					Average values	Average values
	1	2	3	4	5		
	%	%	%	%	%	%	%
Arginine	101.1	101.2	100.5	99.6	101.2	100.7	101.2
Histidine	101.3	100.8	100.0	99.6	100.7	100.5	100.2
Isoleucine	101.1	100.5	99.3	98.8	99.8	99.9	100.0
Leucine	100.3	100.0	99.6	98.5	100.3	99.7	99.9
Lysine	100.1	100.4	98.9	98.8	100.2	99.7	100.5
Methionine	100.0	100.9	99.4	99.5	100.7	100.1	100.7
Phenylalanine	99.7	99.7	98.3	97.6	98.7	98.8	100.8
Threonine	99.7	101.6	98.6	98.2	100.5	99.7	100.8
Tryptophane	99.0	100.2	98.5	97.9	100.3	99.2	100.1
Valine	99.8	100.2	99.1	97.6	100.2	99.4	100.2
Total nitrogen	100.4	101.6	99.7	99.1	99.7	100.1	

Quite different results were obtained with a sample of cottonseed flour [2]. Marked variations in availability of individual amino acids were observed for this foodstuff (table 5). Although 93% of the arginine was available to the rat, only about 65% of the lysine was available. The values for the other amino acids ranged within these extremes. It will be noted that the agreement between values in the same experiment was satisfactory and that the average values were

[2] Proflo.

closely duplicated by a 2nd experiment in which a new set of animals was used.

In the case of peanut flour and wheat, a small loss of amino acids was found. In contrast with cottonseed flour, this loss was rather uniformly distributed among the various amino acids in the food. Availability values ranged from 94.8 to

TABLE 5

True availability of amino acids in cottonseed flour

AMINO ACID	EXPERIMENT 1						EXPERIMENT 2
	Rat number					Average values	Average values
	1	2	3	4	5		
	%	%	%	%	%	%	%
Arginine	94.2	93.3	93.0	94.3	93.7	93.7	93.4
Histidine	90.5	88.0	87.2	89.7	89.3	88.9	89.9
Isoleucine	82.9	81.7	81.0	83.7	83.0	82.5	78.2
Leucine	83.9	80.4	78.6	81.6	79.5	78.8	75.4
Lysine	65.7	59.5	62.3	66.1	64.6	63.6	64.5
Methionine	82.3	79.8	77.3	82.4	81.7	80.7	81.6
Phenylalanine	87.1	85.8	85.6	88.0	86.7	86.6	88.6
Threonine	79.4	76.0	77.2	80.4	78.4	78.3	76.6
Tryptophane	87.7	87.7	90.4	90.0	90.7	89.3	90.8
Valine	81.4	79.1	77.0	74.1	81.8	76.7	78.6
Total nitrogen	88.3	87.5	86.7	88.1	87.5	87.6	

99.5% for peanut flour and from 92.8 to 98.8% for wheat (table 6).

Investigations of the type reported here are subject to the criticism that bacterial action in the lower intestine may have altered the distribution of amino acids in the feces. Complete evidence that errors due to this cause are quantitatively insignificant, will require further investigation. It appears unlikely that the high percentage of lysine found in the feces from the animals fed cottonseed flour was due to bacterial synthesis.

The results indicate that in certain cases information on the amino acid content of foodstuffs will be quite inadequate for evaluating the protein unless availability data are also ob-

tained. While close correlation was found between nitrogen and amino acid availabilities for roast beef, peanut meal and wheat, there were marked exceptions in the case of cottonseed meal. Further investigation will be necessary in order to determine whether such variations in individual amino acid availability in a single foodstuff are to be generally expected or whether cottonseed meal and soybean meal (Melnick et al., '46) are unusual exceptions.

TABLE 6

True availability of amino acids in peanut flour and wheat

AMINO ACID	TRUE AVAILABILITY [1]	
	Peanut flour	Wheat
	%	%
Arginine	99.5	96.4
Histidine	98.8	98.8
Isoleucine	97.2	95.0
Leucine	97.0	95.4
Lysine	97.0	92.8
Methionine	95.8	94.9
Phenylalanine	97.9	96.9
Threonine	94.8	92.2
Tryptophane	97.2	93.2
Valine	95.8	93.2
Total nitrogen	97.7	95.0

[1] Average values based on groups of 5 animals each.

It is probable that changes in the manufacturing procedure may influence the availability of amino acids in cottonseed products. All of the materials studied in this investigation were finely ground. The extent to which this may have influenced amino acid availability is not known.

SUMMARY

1. It was found that all 10 of the essential amino acids in roast beef are completely available to the rat.

2. Wide variations occurred in the availability of the individual amino acids in cottonseed flour. Lysine in this

protein source was only 65% available. The corresponding value for arginine in the same sample was 93%.

3. The availability of each of the essential amino acids in wheat and peanut flour was found to be relatively high. For wheat the values ranged from 92.2 to 98.8%, and for peanut flour from 94.8 to 99.5%.

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VITAMIN B_c, INOSITOL, AND NICOTINIC ACID IN THE NUTRITION OF THE TURKEY[1]

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It is well established that turkey poults require vitamins A, D, E, and riboflavin, pyridoxine, pantothenic acid, nicotinic acid, choline, and vitamin B_c. They probably need vitamin K and they undoubtedly require thiamine, though so far as we are aware, no data on these points have been published, and it has been reported that they need inositol.

Richardson, Hogan and Kempster ('45) diluted a practical ration with a synthetic diet and observed that turkey poults which consumed it developed a spastic cervical paralysis. If the ration was not changed, death usually followed within 2 or 3 days. If pteroylglutamic acid was added to the diluted ration, the poults grew normally and, except for isolated cases of perosis, they were free from abnormalities. Jukes, Stokstad and Belt ('47), Russell, Taylor and Derby ('47) and Schweigert, German, Pearson and Sherwood ('48) confirmed the observations of Richardson, Hogan and Kempster. Jukes et al. noted that when the diet was deficient in pteroylglutamic acid, the erythrocytes were somewhat larger and more elongated than is normal. They estimated that the poult requires 80 μg of the vitamin per 100 gm of ration. Russell, Taylor and Derby arrived at a much higher estimate, 200 μg %. In

[1] Contribution from the Missouri Agricultural Experiment Station, Journal Series no. 1102. The data in this manuscript were taken from a thesis submitted by Mrs. Lance in partial fulfillment of the requirements for the A.M. degree. Aided by a grant from Parke, Davis and Company.

1 group of 8 poults that survived on 150 μg % of pteroylglutamic acid, there were 4 cases of cervical paralysis. There was no paralysis in the group that received 200 μg %. Schweigert and coworkers reported that 80 μg % is insufficient for poults when the eggs from which they hatched were laid by hens that consumed a ration which contained only 42 μg % of the vitamin. Poults from this same group grew normally and had a low mortality rate when their diet contained 200 μg %. However, when the ration of the hens contained a liberal amount of pteroylglutamic acid, their poults responded as well when the diet contained 80 μg % of pteroylglutamic acid as when it contained 200 μg %. Three poults, from hens on their low PGA diet, developed cervical paralysis on a diet that contained 20 μg % of pteroylglutamic acid. None of the poults developed this symptom when their diet contained 80 μg % of the vitamin.

Briggs ('46) observed a high incidence of perosis in poults that received insufficient nicotinic acid and concluded that the minimum requirement is at least 3 to 5 mg per 100 gm of diet. His data indicated that the diet may have been improved by adding 10 mg of this vitamin to his basal diet. Jukes, Stokstad and Belt ('47) confirmed the report by Briggs, and noted no perosis in poults when the diets contained either 5 or 10 mg of niacinamide per 100 gm of food. Jukes et al. ('47) also reported that the poult requires inositol. When this substance was omitted from the diet, growth was retarded, a mild normocytic anemia developed, and some of the erythrocytes had abnormal nuclei. Patrick and associates ('43) reported that the incidence of perosis in poults was not reduced by including inositol in the diet and they apparently found no evidence that the poult requires that substance.

EXPERIMENTAL

The routine followed in the management of experimental turkey poults was described by Richardson, Hogan and Kempster ('45). Most of the poults were Standard Bronze, the others were Broadbreasted Bronze. The experimental periods

were 4 weeks in length and the weights of the poults and the incidence and degree of perosis were recorded at intervals of 1 week. A few of the poults were killed before the end of the experimental period either because they were crippled with perosis or because of infection. In most trials, the mortality rate was unduly high and seemed to have no relation

TABLE 1

Composition of the basal diet

	%		%
Casein	35	Wood pulp	3
Gelatin	10	Lard	10
Cerelose	37	Salts [1]	5
	Vitamins [2] per 100 gm of diet		
Vitamin A	2000 I.U.	Pyridoxine hydrochloride	1 mg
Vitamin D	290 AOAC U.	Ca-pantothenate	3 mg
Vitamin E	2.5 mg	Nicotinic acid	5 mg
2-methyl-1,4-naphthoquinone	2.5 mg	Choline chloride	400 mg
Thiamine hydrochloride	1 mg	Biotin	0.02 mg
Riboflavin	1 mg	Inositol	100 mg
	Pteroylglutamic acid,[3] variable		

[1] Richardson and Hogan ('46).

[2] Vitamins A and D were purchased as a concentrate from Mead Johnson and Company, Evansville, Indiana. The pteroylglutamic acid was generously supplied by Dr. T. H. Jukes, Lederle Laboratories, Pearl River, New York. All other vitamins were generously supplied by Dr. D. F. Green of Merck and Company, Inc., Rahway, New Jersey.

[3] Extinction coefficients of the sample we used, observed in this laboratory by Dr. B. L. O'Dell, indicated a purity of 95% according to the values of Bloom et al. ('44), and of 100% according to the values of Stokstad, Hutchings and Subbarow ('46).

to the adequacy of the diet. It was necessary to ship the poults in from some distance and it is believed that delays in transit, with exposure to cold and deprivation of food and water, had reduced the vitality of the weaker poults to a low ebb. The rations, described in table 1, were of the simplified type. The casein, prepared in the laboratory from skim milk, was precipitated with hydrochloric acid and extracted

thoroughly with dilute acetic acid. The composition of the basal diet was modified at times in minor respects in attempts at improvement, but since the modifications proved to be of no consequence they have been ignored and the data on similar diets have been combined. The basal diet most frequently used is described in table 1.

The amount of pteroylglutamic acid required by the turkey poult

The growth rates reported in table 2 were highly variable but the weights were inferior when the rations contained less

TABLE 2

The amount of pteroylglutamic acid required by the turkey poult

GROUP [1]	1	2	3	4	5	6	7
Pteroylglutamic acid (mg/100 gm)	0.01	0.025	0.05	0.1	0.15	0.2	0.3–0.4
No. of separate trials	2	1	4	6	1	12	8
No. of poults	20	10	39	54	6	107	74
No. of poults killed [2]	0	0	0	2	0	7	7
Mortality (%)	75	70	51	18	17	22	4
Hematocrit [3] reading (%)	32	35	35	33	36	38	36
Hemoglobin [3] in blood (%)	7.4	8.2	8.5	6.8	6.9	8.4	8.7
Perosis (%)	0	0	7.7	9	17	7.4	12
Perosis (score)	0	0	50	55	25	85	60
Cervical paralysis (%)	35	50	16	3.7	0	0	0
Broken feathers (%)	100	100	53	19	60	8	6
Broken feathers (score)	3.7	3.5	3.4	2	3	1.3	1
Avg. weight at 4 weeks (gm)	277	198	332	373	334	501	451

[1] As a rule the diets contained 100 mg % of inositol but the exceptions are not shown separately since they had no appreciable effect on the results.

[2] Perosis or diarrhea.

[3] Taken at 3 weeks.

than 0.2 mg % of the vitamin. The poults grew as rapidly when the diet contained 0.2 mg % of the vitamin as when it contained larger amounts. The poults that received 0.2 mg % had an average weight of 501 gm at 4 weeks. We were unable to separate the sexes at that age, but presumably the

number of males and females was about the same. No attempt was made to search the literature for maximum growth rates, but the highest weight at 4 weeks recorded in our files was approximately 500 gm for males. Turkey poults grow rapidly on synthetic diets that are properly constructed.

When the diet contained less than 0.1 mg % of the vitamin, the mortality rate was increased, and of the 54 poults that received the diet which contained 0.1 mg %, 2 developed typical cervical paralysis. As the amount of the vitamin was reduced, the incidence of paralysis increased.

When the poults were 3 weeks old the hematocrit volume was determined by the method of Van Allen ('25). The blood for hemoglobin determinations was drawn from the ulnar vein and 0.05 ml was hemolyzed in 10 ml of distilled water. Two drops of concentrated ammonium hydroxide were added and the mixture was centrifuged for 20 minutes. Centrifugation was completed within 10 minutes after the ammonia had been added. A Pfaltz and Bauer protoelectric colorimeter, equipped with a 540 filter, was used for the hemoglobin determinations. With the aid of a standard response curve the deflection of the galvanometer was converted into percentage of hemoglobin in the blood.

The percentage of hemoglobin in the blood and the red cell volume were determined for each poult included in table 2, and the poults which received either 0.1 or 0.15 mg % of pteroylglutamic acid were mildly anemic. It was unexpected that these poults would be more anemic than were those that received still smaller amounts of vitamin B_c and presumably this was due to the fact that the more susceptible poults in groups 1, 2, and 3 had died before the determinations were made. This difference was not reported by Russell, Taylor and Derby ('47), possibly because their observations were made when the poults were older. There was no correlation between the incidence of perosis and the amount of vitamin B_c in the diet. The percentage of perosis shown in table 2 is the per cent of poults that developed the abnormality. The "degree of perosis" is an empirical rating of its severity. Mild

cases with swollen joints were marked 25. Moderate cases with markedly swollen joints and 1 foot turned to the side were marked 50. Severe cases in which the tendon of Achilles had slipped from the chondyle were marked 75. The most severe cases in which the poults had lost control of both legs were marked 100. It will be noted in table 2 that the incidence of perosis was not reduced when the amount of pteroylglutamic acid in the diet was raised to 0.3 mg %. Presumably this diet contained ample amounts of all recognized nutrients that are required to prevent perosis. The occurrence of the abnormality under these circumstances is regarded as evidence that the diet is still deficient in an unrecognized essential nutrient.

Several investigators have reported that the feathers are imperfect when chicks receive insufficient pteroylglutamic acid, and Russell, Taylor and Derby ('47) had the same experience with turkeys. Our observations on this point are described in table 2. The percentage of broken feathers is the percentage of poults affected. The score indicates the degree of damage. A score of 0 indicates perfect feathers and a score of 4 indicates the most extreme damage. It will be noted that when the diet contained less than 0.2 mg % of pteroylglutamic acid, the percentage of affected poults was high and the damage was extensive. When the diet contained 0.2 mg % or more of the vitamin, the condition of the feathers was vastly improved, but still they were not perfect. The close confinement may have contributed to the imperfections, but to us it seems probable that the synthetic diets were not entirely adequate and that a mild nutritional deficiency is the explanation of the minor defects in groups 6 and 7 of table 2.

Our data show that the minimum requirement of the turkey poult for pteroylglutamic acid is more than 0.15 mg % of the diet but not over 0.2 mg %. It may be, though, that the minimum requirement depends in some degree on the other constituents of the diet as was reported for chicks by Luckey, Moore, Elvehjem and Hart ('46). It is also possible that the minimum requirement depends in some degree on the previous history of the poults, as reported by Schweigert, German,

Pearson and Sherwood ('48). It seems reasonable, though, that the minimum allowance recommended for commercial practice should meet the maximum requirement.

The results of a more intensive study of the cellular constituents are summarized in table 3. The erythrocytes were counted by the method of Wiseman as described by Biester and Devries ('43, chap. 4, p. 69), and the leucocytes were counted by the method of Blain ('27–'28). The 2-slide method

TABLE 3

Observations on cellular constituents of the blood

GROUP	1	2	3	4[1]
Pteroylglutamic acid (mg/100 gm)	0.10	0.15	0.20	
No. of poults in group	8	5	13	10
Avg. wt. of poults at 4 weeks (gm)	265	334	440	432
Hemoglobin in blood (%)	6.1	6.9	8.9	9.2
Hematocrit reading (%)	30	36	42	40
Erythrocytes,[2] 10^6 per mm^3	1.82	1.61	2.43	2.25
Leucocytes, 10^3 per mm^3	12.8	17.2	20.6	24.8
Lymphocytes, 10^3 per mm^3	5.965	10.303	9.085	11.855
Heterophils, 10^3 per mm^3	6.016	5.710	10.332	11.507
Eosinophils, 10^3 per mm^3	0.128	0.258	0.241	0.307
Basophils, 10^3 per mm^3	0.371	0.516	0.812	0.794
Monocytes, 10^3 per mm^3	0.294	0.396	0.127	0.248
Thrombocytes, 10^3 per mm^3	18.230	17.450	33.400	36 680

[1] Practical control ration.

[2] All cellular constituents were counted on 5 poults in each group.

was used in making the smears for the differential white cell counts.[2] The blood was taken from the ulnar vein and the first drop was always discarded. The films were stained with Wilson's blood stain (200 mg of Wilson's stain dissolved in 50 ml of acetone-free methyl alcohol). After drying the blood film in the air, without previous fixation, the smear was covered with an excess of the stain. One and one-half minutes after the addition of the stain neutral distilled water was added, in a quantity equal to that of the staining fluid. The

[2] We are indebted to Dr. H. C. McDougle who developed the method and taught us how to use it.

mixture was allowed to stand for about 2 minutes (until a greenish film had formed on top of the staining mixture) and the preparation was washed with neutral distilled water for about 30 seconds, or until the thinner portions of the film became a bluish-purple in color. Schmeisser's method ('15) was used in making the classifications. Three hundred white blood cells were counted in each smear. Groups 2 and 3, which received 0.1 and 0.15 mg % of vitamin B_c, developed a mild macrocytic anemia. The erythrocytes also developed other abnormalities, including anisocytosis, poikilocytosis and mottled and abnormally shaped nuclei. The poults that received 0.2 mg % had about the same erythrocyte count, hematocrit reading and percentage of hemoglobin in the blood, as did the poults that consumed the practical control ration. When the ration contained 0.1 mg % of vitamin B_c there was a marked reduction in the number of thrombocytes and leucocytes, including both lymphocytes and granulocytes. When the ration contained 0.15 mg % of vitamin B_c, the blood still had only one-half the normal number of thrombocytes, but the average number of leucocytes was only slightly depressed. Two of the 5 poults examined had a granulocyte deficiency. The leucocytes were also enlarged, with mottled and abnormally shaped nuclei. When the ration contained 0.2 mg % of pteroylglutamic acid, as in group 3, there was a slight but consistent decrease in the number of both thrombocytes and leucocytes. These counts leave the impression that even though the synthetic diets support an excellent rate of growth they are still slightly inadequate. There were some inconsistencies between groups, but presumably these could be explained by the variability of the poults at the time they reached the laboratory.

The requirement of the turkey poult for inositol

Our data on this point are shown in table 4 and give no definite indication that the amount of inositol in the diet has any influence on the weight, the number of red cells, the percentage of hemoglobin in the blood, or the differential cell

counts. However, when inositol was omitted from the diet, the leucocyte counts were lower than when it was added and the difference was statistically significant. Jukes and associates ('47) reported that when turkey poults received insufficient inositol they were mildly anemic and the erythrocytes were slightly abnormal. It is our view that the abnormality was due to a slight inadequacy in the supply of pteroylglutamic acid. Jukes et al. did not report leucocyte counts.

TABLE 4

The requirement of the turkey poult for inositol

GROUP [1,2]	1	2	3	4
Inositol (mg/100 gm)	100	0	100	0
No. of poults	10	6	7	7
No. of poults killed [3]	0	0	2	0
No. of poults survived	8	6	5	5
Hematocrit reading (%)	43	43	41	40
Hemoglobin in blood (%)	9.2	9.6	8.6	8.4
Erythrocytes,[4] 10^6 per mm^3	2.47	1.97	2.38	2.38
Leucocytes,[4] 10^3 per mm^3	21.6	16.4	19.6	15.4
Thrombocytes,[4] 10^3 per mm^3	32.02	25.66	34.78	34.28
Perosis (%)	30	17	0	14
Perosis (score)	75	25	0	50
Avg. weight at 4 weeks (gm)	452	408	428	489

[1] The basal ration contained 0.2–0.3 mg pteroylglutamic acid per 100 gm.

[2] Groups 3 and 4 were under observation at a later date than those in groups 1 and 2. They are shown separately in order to illustrate the variability in weight.

[3] Perosis or diarrhea.

[4] The cellular constituents were counted on 5 poults in each group.

The relation between nicotinic acid and the incidence of perosis

None of our diets contained less than 5 mg % of nicotinic acid, which is more than enough to prevent any specific symptoms of a deficiency. Briggs ('46) and Jukes and associates ('47) have presented evidence which indicates a relation between nicotinic acid and the incidence of perosis. Our data, presented in table 5, indicate that when the amount of nicotinic acid added to the basal synthetic diet was increased

from 5 to 10 mg % the incidence of perosis, and the severity of the abnormality, were decreased by about 50%. However, the syndrome was never completely prevented in poults which grew rapidly, which suggests that an unexplained factor is involved in the abnormality.

TABLE 5

The relation between nicotinic acid and the incidence of perosis

GROUP [1]	1	2
Nicotinic acid (mg/100 gm)	5	10
No. of separate trials	6	6
No. of poults	57	55
No. of poults killed [2]	4	2
Mortality (%)	12	7
Hematocrit reading (%)	37	37
Hemoglobin in blood (%)	8.5	8.7
Perosis (%)	19.3	7.3
Perosis (score)	87	58
Avg. weight at 4 weeks (gm)	445	467

[1] The basal ration contained 0.2–0.3 mg pteroylglutamic acid per 100 gm.
[2] Perosis or diarrhea.

SUMMARY AND CONCLUSIONS

1. The amount of pteroylglutamic acid required by the turkey poult for maximum growth and normal development is approximately 0.2 mg %.

2. Under our experimental conditions the addition of inositol had no appreciable effect on either growth or the differential leucocyte count. When inositol was omitted from the diet the number of leucocytes was subnormal.

3. The incidence of perosis in turkey poults was decreased by increasing the nicotinic acid content of the diet from 5 to 10 mg %. Perosis was never completely absent in spite of the fact that the rations contained all factors known at present to prevent it.

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THE VITAMIN A CONTENT OF HUMAN BLOOD PLASMA AS AN INDEX OF CAROTENE UTILIZATION [1]

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TWO FIGURES

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The biological activity of the carotenoid pigments apparently depends upon the ability of the animal body to convert these pigments into vitamin A. Guggenheim ('44), Fraps and and Meinke ('45) and Kemmerer and Fraps ('45) in experiments with rats have shown that the carotenes from different food sources are not equally well utilized. In a series of experiments with human subjects, Booher, Callison and Hewston ('39) and Booher and Callison ('39) have also shown that carotenes from various sources have different biological values. Their investigations also demonstrated that the human subject is less efficient than the rat in the utilization of carotene as a source of vitamin A. It appears, therefore, that neither chemical analysis nor biological assay by the rat-growth method of foods containing carotene can be depended upon as a measure of the vitamin A value for human beings.

The investigation of the utilization of carotenes is made difficult, as compared with the water-soluble vitamins, by the fact that vitamin A is stored in the liver in relatively large

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amounts and may be retained for long periods of time. Subjects must, therefore, be depleted of their reserve stores of vitamin A before any experimental work can be carried out. An increase in the dark adaptation time (night blindness) has generally been used as the criterion for determining the point at which the reserve vitamin A has been exhausted. The dark adaptation time has also been used for measuring the response to vitamin A supplementation. This method has, however, been criticized on the basis of non-specificity. Also the studies of Bodansky, Lewis and Haig ('41) and Lewis, Bodansky, Falk and McGuire ('42) provide data which lead to the conclusion that the level of vitamin A in the blood plasma may be a more sensitive index of vitamin A-status than the dark adaptation time.

The following study was carried out in an effort to determine the feasibility of using the level of vitamin A in the blood plasma as a measure of the utilization of carotenoids by humans.

EXPERIMENTAL

The general plan of the experiment was to place a human subject on a vitamin A-low diet until the level of vitamin A in the blood plasma dropped significantly. The individual would be standardized by determining the changes in the blood level resulting from the administration of known amounts of vitamin A. The subject could then be used to measure the efficiency of the utilization of carotenoids by measuring the level of vitamin A in the blood after the administration of known amounts of the test food. The subject studied was a female, 34 years old and in good general health.

Experimental periods

The experiment which lasted over 2 years was divided into 3 periods.

1. Preliminary period. This lasted for 49 days, with the subject on a normal diet, during which the carotene and vitamin A of the blood were determined at frequent intervals in order to have a measure of the normal levels for this subject.

2. Depletion period. The subject received a vitamin A-low diet in order to exhaust the reserve supply of vitamin A. This period covered 598 days, but included 2 rather extended periods of interruption. The time was divided as follows: depletion — 166 days; interruption — 72 days; depletion — 81 days; interruption — 164 days; depletion — 115 days. The total number of days actually on the depletion diet was 362. The periods of interruption were not planned as part of the experiment, but were the result of unexpected circumstances which caused the subject to be away from the laboratory for these periods.

3. Experimental period. This was 112 days, during which the subject remained on the depletion diet, but received supplements of vitamin A or papaya.

Diet

The vitamin A-low diet, which was estimated to contain 100–200 I.U. vitamin A per day, was similar to that of Booher et al. ('39). A vitamin capsule containing vitamins of the B-complex and ascorbic acid was taken daily and an iron tablet was taken 3 times each week. This diet is referred to as the basal diet.

During periods when the experiment was interrupted, the subject ate a normal diet except that foods unusually rich in vitamin A activity such as liver, carrots and spinach were avoided. This diet is referred to as the normal-restricted diet.

No attempt was made to regulate the dietary intake of vitamin E and no supplements of this vitamin were taken during the depletion period. During the periods of vitamin A supplementation, 100 mg of a vitamin E concentrate [3] containing 34% natural mixed tocopherols were administered with the vitamin A supplement. Thus the possibility of variation in the utilization of the vitamin A of the supplements due to the influence of vitamin E was removed, since this factor was always present in adequate amounts.

[3] Distillation Products, Inc., Rochester, N.Y.

Determination of blood levels

The levels of vitamin A and carotene in the blood were determined at frequent intervals during the preliminary period, at least once a week during the depletion period, and at least twice a week during the experimental period. The blood samples, which were taken in the morning, were analyzed for both vitamin A and carotene according to the method of Kimble ('39) using 5 ml of plasma. The factors used to convert the L values obtained with the Evelyn colorimeter to International Units of vitamin A and μg of carotene and also the factor for correcting L-620 for the presence of carotene were determined in this laboratory. For the determination of the carotene factor, a sample of crystalline carotene containing 90% β-carotene and 10% α-carotene [4] was used. A sample of distilled vitamin A esters [5] which had been especially prepared for use as a standard was used to determine the factor for vitamin A.

Supplements

The supplements of vitamin A were administered in the form of a distilled vitamin A concentrate suitably diluted with cottonseed oil. The original concentrate and the diluted samples, which were freshly prepared each week, were assayed chemically by means of the antimony trichloride reaction.

The papaya supplements were given in the form of a frozen puree prepared with the aid of a Waring Blendor. A large volume was well-mixed and then placed in half-pint packages and quick-frozen. Two separate lots were prepared and held in a freezing locker until used. The carotene content of each of these lots was determined colorimetrically with the Evelyn colorimeter. Samples of frozen papaya were extracted according to the procedure of Moore and Ely ('41). The extracts were saponified with alcoholic KOH at room temperature and the carotenes were transferred to petroleum

[4] S. M. A. Carotene.
[5] Distillation Products, Inc., Rochester, N. Y.

ether. The washed and dried extracts were chromatographed on Hyflo Super Cel (Wilkes, '46) and separate determinations were made of β-carotene and cryptoxanthin, the 2 biologically active carotenoids found to be present in the papaya. The values obtained were converted to International Units of vitamin A on the basis that 0.6 μg β-carotene or 1.2 μg cryptoxanthin equals 1.0 I.U. of vitamin A activity. The average values for the 2 samples were 1230 and 1500 I.U. per 100 gm, of which about 80% was contributed by cryptoxanthin.

RESULTS AND DISCUSSION

The changes in the levels of vitamin A and carotene in the blood which occurred during the depletion period are shown in figure 1. The average and range of 9 values determined during the 49 days of the preliminary period were 133 ± 10 I.U. vitamin A and 149 ± 25 μg carotene per 100 ml of plasma.

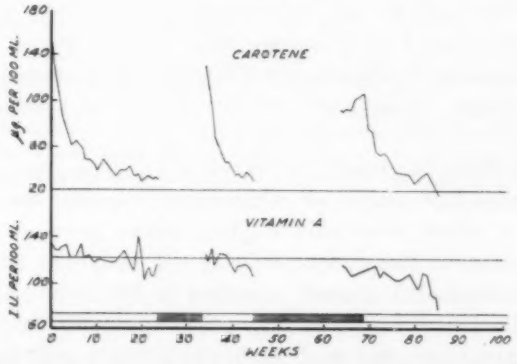

Fig. 1 The levels of vitamin A and carotene in the blood during the depletion period. The bar along the bottom of the chart indicates the type of basal diet: open spaces — vitamin A deficient diet; lined spaces — more liberal diet, but containing no foods very rich in vitamin A.

The change from a normal diet to the vitamin A-low diet resulted in an immediate and sharp drop in the carotene level of the blood. This occurred, as expected, each of the 3 times that the change was made from a normal to the vitamin A-

low diet. The vitamin A level, on the other hand, showed no consistent change during the first 140 days, but maintained a level between 118 and 140 I.U. per 100 ml. After the first 140 days a slight drop occurred and the level fluctuated between 102 and 117 I.U. per 100 ml for the next 24 days. At this time, however, the experiment was interrupted and the subject changed to the normal-restricted diet. After 72 days the subject returned to the basal diet for 81 days during which there was a slight but definite drop, as shown on figure 1, from about 120 I.U. vitamin A per 100 ml to about 110 I.U. per 100 ml. After a second interruption (164 days), the subject returned to the basal diet with a blood plasma vitamin A level of about 115 I.U. per 100 ml. The level then dropped gradually over a period of 106 days. At the end of this time a relatively sharp drop occurred to a level below 90 I.U. vitamin A per 100 ml of plasma. Nine days later the blood level was 76 I.U. per 100 ml — about 60% of the normal value for this subject. The subject was, therefore, considered to be sufficiently depleted and supplements were begun. There were no other symptoms or evidences of vitamin A depletion such as loss of weight, roughness of the skin or subjective night blindness.

Graded doses of vitamin A esters in oil were given in order to determine the amout of vitamin A required per day to maintain a blood level only slightly below normal. The object was to meet the daily needs of the body but to prevent the accumulation of reserve supplies in the liver so that the level in the blood would be dependent upon the intake. The amounts given per day and the blood levels found are shown in figure 2. The dosage was gradually increased from 2000 I.U. to 3500 I.U. per day over a period of 28 days, during which there was first a leveling off and then an increase in the level of vitamin A in the blood. At the end of 32 days, however, there occurred an unexpected drop in the blood level. The dosage was, therefore, raised to 10,000 I.U. per day and continued at that level for 7 days. During that time the subject developed a cold with a temperature of 100°F. She was then

given a more liberal diet plus supplements of from 10,000 to 100,000 I.U. vitamin A per day for 3 days. But, in spite of these high doses of vitamin A, the blood level continued to fall and reached the low level of 46 I.U. per 100 ml at the end of the 3 days. At this point the subject was given 100,000 I.U. vitamin A plus about 200 gm of cooked liver. This resulted in an increase in the plasma vitamin A to a value of 126 I.U. per 100 ml on the following day.

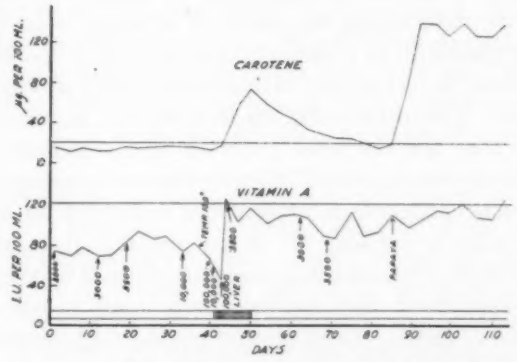

Fig. 2 The levels of vitamin A and carotene in the blood during the experimental period. The numbers with the arrows indicate I.U. of vitamin A administered per day. The bar along the bottom of the chart indicates the type of basal diet: open spaces — vitamin A deficient diet; lined spaces — more liberal diet.

For the next 6 days the subject continued to receive a more liberal diet plus 3500 I.U. per day. During this time the plasma level was maintained at approximately 110 I.U. vitamin A per 100 ml. The experiment was, therefore, resumed and the subject was returned to the basal diet plus a supplement of 3500 I.U. vitamin A per day. This dosage was continued for 11 days during which the plasma level averaged 111 I.U. per 100 ml with a range of 103 to 118. The dosage was then reduced to 3000 I.U. per day for 8 days and the blood level dropped to 90 I.U. per 100 ml. The level gradually rose again to about 100 I.U. per 100 ml when the dosage was restored to 3500 I.U. per day for 16 days. It was concluded from these

data that 3500 I.U. vitamin A per day were required by this subject in order to maintain the vitamin A in the plasma at a level of 100 to 110 I.U. per 100 ml.

Supplements of papaya were then given in amounts calculated, on the basis of the chemical assays, to provide 3500 I.U. of vitamin A activity per day. These supplements were fed over a period of 28 days during which the average level of vitamin A in the plasma rose slightly and averaged 112 I.U. per 100 ml. Thus the supplements of papaya were just as effective in maintaining the level of vitamin A in the blood as the supplements of vitamin A esters in oil — perhaps even more so since the trend was rather consistently upward during this period. This interpretation is, however, based on the assumption that the chemical analysis of the papaya samples quantitatively measured all the pro-vitamin carotenoids present. Since cryptoxanthin contributed over 80% of the calculated vitamin A activity of these samples, it may also be concluded that cryptoxanthin was well-utilized by this subject.

This experiment was unnecessarily long because of the interruptions during the depletion period and because of the unexpected drop in the plasma level which occurred in the middle of the experimental period, probably as the result of the subject's catching a cold. Clausen ('43) and Brenner and Roberts ('43) have reported that marked decreases occur in the level of vitamin A in the blood of subjects with infections or fever. Also this subject was probably depleted more than was necessary. The experimental period probably could have been started at the end of the first 166 days.

This experiment has demonstrated that the blood level can be used as an index for the study of the utilization of carotenoids. The level of vitamin A in the plasma of a depleted subject was shown to increase or decrease as the amount of vitamin A or carotene ingested increased or decreased. The data obtained, however, do not indicate that the blood plasma level is more sensitive than the dark adaptation time as an index of vitamin A nutrition although it is undoubtedly a more specific measure.

SUMMARY AND CONCLUSIONS

A single subject was placed on a vitamin A-low diet for 3 periods of 166, 80 and 115 days, respectively. Between these 3 periods were 2 periods of moderate vitamin A intake of 73 and 163 days, respectively.

The level of carotene in the plasma dropped rapidly each time the change was made to the vitamin A-low diet. The vitamin A level, on the other hand, did not show any significant change for 140 days, then dropped very gradually over a long period of time and finally made a rather abrupt drop. This drop was taken to indicate the end of the depletion period. The plasma levels were 149 μg carotene and 133 I.U. vitamin A per 100 ml when the experiments began and 15 μg carotene and 76 I.U. vitamin A per 100 ml at the end of the depletion period.

The feeding of increasing amounts of vitamin A (distilled esters in oil) resulted in a gradual increase in the vitamin A level in the plasma. An intake of 3500 I.U. per day maintained a level of 100 to 110 I.U. per 100 ml in the plasma and was taken to be a satisfactory intake for maintenance for this subject. Amounts of papaya which, according to chemical assay, provided 3500 I.U. of vitamin A activity per day in the form of carotenoids were then fed for 28 days. The plasma level of vitamin A was maintained between 100 and 120 during this period showing that the carotenoids of the papaya were well-utilized by this subject. Cryptoxanthin contributed 80% of the estimated vitamin A activity of the papaya samples.

These results show that the plasma level of vitamin A can be used as a measure of the utilization of carotenoids by human beings. The data obtained, however, do not indicate that the blood plasma level is more sensitive than the dark adaptation time as an index of vitamin A nutrition, although it is undoubtedly a more specific measure.

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THE EFFECTS OF SOYA LECITHIN ON THE ABSORPTION, UTILIZATION AND STORAGE OF VITAMIN A AND CAROTENE IN THE WHITE RAT

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INTRODUCTION

It has been reported by several investigators (McCoord and Luce-Clausen, '34; Davies and Moore, '34; Bauman et al., '34; LePage and Pett, '41) that the percentage utilization of dietary vitamin A and carotene is small, due to poor absorption and considerable destruction. Hickman et al. ('42), Sherman ('42), and others have demonstrated that the destruction of these constituents can be partially inhibited by the simultaneous ingestion of alpha-tocopherol and other fat soluble antioxidants that are normally present in certain fats and oils. Adlersberg and Sobotka ('43a) reported that in sprue the ingestion of vitamin A did not result in an increase in the blood level, but when lecithin was given at the same time there was a moderate rise in the serum vitamin A. In normal individuals, they (Adlersberg and Sobotka, '43b) found a rise of 41% in the blood 4 hours after a test dose of 18,000 I.U. of vitamin A. When lecithin was fed in addition, the rise in blood vitamin A was of the order of 200%. Slanetz and Scharf ('43, '45) observed a greater growth response when lecithin was fed to vitamin A-depleted rats along with vitamin A and carotene. They demonstrated also that lecithin mark-

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edly influenced both liver storage and blood levels of vitamin A. These authors have postulated that lecithin contains an unknown factor which influences carotene and vitamin A utilization in the rat.

In a previous contribution from this laboratory (Esh et al., '47), it was observed that when lecithin was fed to cows along with vitamin A during the later stages of the gestation period, more vitamin A was transferred to the colostral milk as well as to the newborn calves by way of the placenta. Moreover, when a mixture of lecithin and vitamin A was added to a skim milk ration of newborn calves, they grew quite well and the blood vitamin A and liver storage were comparable to those of colostrum-fed calves.

In the investigation reported herein, a study was made in rats of the effect of feeding lecithin during the vitamin A depletion period and during the vitamin A and carotene supplementation periods on the rate of growth, plasma vitamin A level and liver storage of vitamin A.

EXPERIMENTAL

Experiment I. Soya lecithin and vitamin A

Fifty young albino rats reared under comparable conditions in our breeding colony were allotted to this experiment at weaning (24 days of age). Their weights varied from 48 to 52 gm. They were divided into 2 groups of equal sex and litter distribution and depleted of vitamin A on rations (a) and (b) (see table 1).

The rations were freshly prepared each week and stored in a refrigerator. The rats were maintained in individual cages and the rations provided ad libitum for 39 days. Weights were recorded weekly for the first 3 weeks and twice weekly thereafter. The same feeding and management routine was followed in each of the 3 experiments. Four rats were sacrificed from each group at the end of 3 weeks and a second set of 4 from each group at the end of the depletion period (39 days). The blood from each group of 4 animals was pooled

for serum vitamin A analysis and the livers obtained for individual liver vitamin A storage determination.

At the end of the depletion period the animals were regrouped as shown in table 2, experiment I. This regrouping was done to determine whether there was a carry-over effect of the depletion diet to the period of vitamin A supplementation. At this point vitamin A supplementation began.

TABLE 1

Percentage composition of the basal rations used

INGREDIENT	RATION				
	a	b	c	d	e
Corn starch	50	50	65	65	65
Commercial dextrose (Cerelose)	17	17	..	..	..
Casein, vitamin free	18	18	18	18	18
Hydrogenated vegetable fat (Crisco)	10	8	12	10	11.93
Crude soya lecithin [1]	..	2	..	2	..
Choline chloride	..	..	..	..	0.07
Salt mixture [2]	4	4	4	4	4
Yeast extract [3]	1	1	1	1	1
Distilled water	Ad libitum				

[1] "Centrol II." Lecithin from Central Soya Company, Inc., containing 65% phosphatides suspended in 35% soybean oil. The phosphatides consist of approximately 50% lecithin and 50% cephalin.

[2] U.S.P. XII mixture no. 2 for vitamin A-free ration, to which were added traces of KI and $CuSO_4$.

[3] Yeast extract supplied through the courtesy of Mead Johnson and Co., Evansville, Indiana.

A concentrate of the natural vitamin A ester was diluted with cottonseed oil and 5 μg of the vitamin administered to each rat daily. The vitamin A feeding was continued for 21 days. The rats were then sacrificed, the blood from each group pooled for serum vitamin A determination, and the livers obtained for individual liver vitamin A storage estimation.

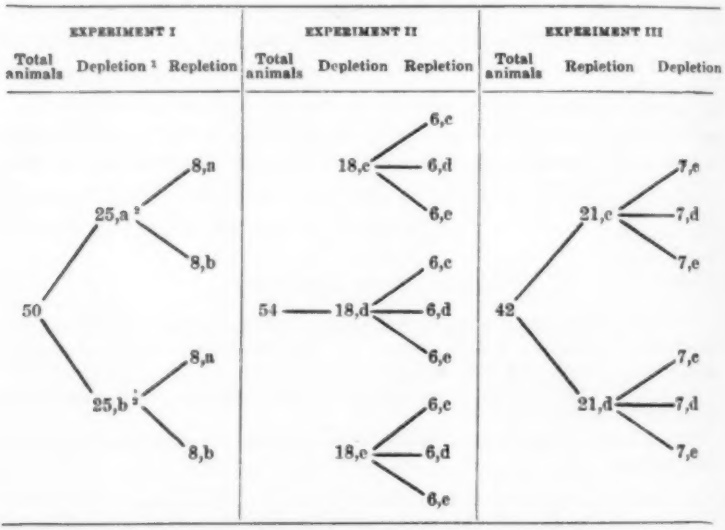
Experiment II. Soya lecithin, choline and vitamin A

Since choline is a physiologically important group in the lecithin molecule, the results obtained in experiment I

prompted an investigation of the effect of choline on the absorption and liver storage of vitamin A. Fifty-four weanling albino rats from the same breeding colony, and raised under similar conditions, were divided into 3 groups of equal sex and litter distribution and depleted of vitamin A on rations (c), (d), and (e) (see table 1). The amount of choline included

TABLE 2

Distribution of the experimental animals in the various dietary groups of the 3 experiments



[1] The letter following the number indicates the ration (see table 1).

[2] Four animals from each of these groups were sacrificed at the end of 3 weeks and 4 more at the end of the depletion period. Two died.

in ration (e) was that calculated to be in the lecithin added to ration (d). This amount is substantially greater than that fed by Slanetz and Scharf ('45).

At the end of the depletion period (39 days) each original group was again divided into 3 groups as indicated in table 2 and 5 μg of vitamin A ester administered daily for the next 21

days. At the end of the 21-day repletion period the animals were exsanguinated and the livers obtained for vitamin A assay.

Experiment III. Soya lecithin, choline and carotene

This experiment was undertaken in an attempt to verify the unexpected effect of choline feeding and to study the effect of choline, lecithin and carotene feeding on the liver storage of vitamin A.

Forty-two weanling albino rats were divided into 2 comparable groups and depleted of vitamin A on rations (c) and (d) (see table 1). At the end of the depletion period (39 days) the animals were allotted to 6 sub-groups as indicated in table 2, experiment III. For the next 21 days each rat received a daily dose of 20 μg of β-carotene in cottonseed oil solution. Twenty-four hours after the last carotene feeding the animals were exsanguinated and the livers removed for assay.

Assay-procedure

Blood serum vitamin A was determined by the method of Kimble ('39). Vitamin A was determined on the entire liver by the method of Lewis et al. ('42) with minor modifications.

RESULTS

Experiment I

The rats in the lecithin group made more rapid and greater weight gains during the depletion period. Weights of the animals on the basal vitamin A-free ration remained almost constant after 2 weeks, while the lecithin-fed group continued to gain until near the end of the depletion period. During the second week there was a decrease in the weight of both groups. The cause of this unexpected change is not certain. It is thought to have resulted from the extremely hot weather during the week.

Three rats in the lecithin group showed symptoms of vitamin A deficiency after 4 weeks. Toward the end of the deple-

tion period many of the rats of both groups showed the classic symptoms of avitaminosis A. Two of them died after 35 days.

The results of the vitamin A analyses of the blood sera and livers of the rats sacrificed at the end of 3 weeks and at the end of the depletion period are presented in table 3. It will be noted that the liver reserves were low at the end of 3 weeks and essentially exhausted at the end of the depletion period. Although the data are limited, the results indicate that the liver reserves are exhausted more rapidly when lecithin is fed and this is accompanied by lower blood serum vitamin A values.

During the vitamin A supplement feeding period the rats which received lecithin made greater weight gains, as shown in table 4. The liver storage of vitamin A was also greater in the lecithin-fed group. A statistical analysis of the liver storage data showed significance at the 1% level.

There appears to be a slight carry-over effect of lecithin feeding during the depletion period but this effect was not found to be statistically significant. The serum vitamin A at the end of the repletion period was found to be slightly lower when lecithin was fed. These blood data are too few for statistical analysis. Similar results were obtained in experiment III, however, as shown in table 7.

A preliminary inspection of the data indicated a sex difference in liver storage of vitamin A. This was verified by a statistical analysis of the data for the sexes presented in table 8.

Experiment II

The results of this experiment are summarized in table 5. During the depletion period both the lecithin- and choline-fed groups made greater weight gains than the control (basal) group. An analysis of these data showed the difference between the gains of the lecithin group and the control group to be significant at the 1% level. The difference between the choline group and the control group was not significant.

As in the first experiment, the liver storage of vitamin A was significantly higher (1% level) when lecithin was fed with vitamin A. The liver storage in the choline-fed rats was not significantly different from that in the control group.

TABLE 3

The effect on blood and liver vitamin A of lecithin feeding during vitamin A depletion

DEPLETION RATION	AT THE END OF 3 WEEKS			AT THE END OF THE DEPLETION PERIOD		
	No. of rats	Serum A	Total liver A	No. of rats	Serum A	Total liver A
		μg/100 ml	*μg*		*μg/100 ml*	*μg*
Basal	4	18	1.1	4	15	0.5
Basal + lecithin	4	16	0.6	4	13	0.3

TABLE 4

Effects of lecithin on the rate of growth and liver storage of vitamin A during the feeding of vitamin A (5 μg daily) for 21 days (8 rats in each dietary group)

SUPPLEMENT IN ADDITION TO VITAMIN A	GAIN IN WEIGHT (GM)			VITAMIN A (μG/LIVER)			PERCENTAGE OF VITAMIN A STORED
	Depletion diet			Depletion diet			
	(a)	(b)	Av.	(a)	(b)	Av.	
Lecithin	32.9	33.2	33.0	20.2	22.8	21.5	20.5
None	28.0	32.0	30.0	16 4	18.2	17.3	16.5

TABLE 5

Effects of lecithin and choline on the rate of growth and liver storage of vitamin A during the feeding of vitamin A (5 μg) daily for 21 days (6 rats in each dietary group)

SUPPLEMENT IN ADDITION TO VITAMIN A	GAIN IN WEIGHT (GM)				VITAMIN A (μG/LIVER)				PERCENTAGE OF VITAMIN A STORED
	Depletion diet [1]				Depletion diet [1]				
	(c)	(d)	(e)	Av.	(c)	(d)	(e)	Av.	
Lecithin	33.0	36.0	27.0	32.0	17.0	21.2	18.0	18.7	17.9
Choline	29.0	27.3	22.0	26.1	14.0	12.0	12.7	12.9	12.3
None	26.0	27.0	25.0	26.0	12.2	14.8	13.1	13.3	12 6

[1] See table 1 for description of depletion diets.

Experiment III

The weight gains during the depletion period were so similar to those obtained in experiment I, and in the groups fed the basal ration and the basal ration plus lecithin of experiment II, that the data are not reported. Again the lecithin basal group made significantly greater (1% level) gains. The

TABLE 6

Comparative effects of lecithin and choline on the rate of growth and liver storage of vitamin A and "carotene" during the feeding of carotene (20 μg daily) for 21 days (6 rats in each dietary group)

SUPPLEMENT IN ADDITION TO CAROTENE	GAIN IN WEIGHT (GM)			VITAMIN A (μG/LIVER)			"CAROTENE" (μG/LIVER)			PERCENTAGE OF VITAMIN A STORED
	Depletion diet			Depletion diet			Depletion diet			
	(c)	(d)	Av.	(c)	(d)	Av.	(c)	(d)	Av.	
Lecithin	25.6	26.2	25.9	65.5	67.5	66.5	3.7	2.6	3.1	32.4
Choline	22.0	24.0	23.0	52.9	58.4	55.6	3.7	2.6	3.1	27.2
None	14.3	20.0	17.0	51.7	59.0	55.3	2.7	1.9	2.3	26.8

TABLE 7

Comparative effects of lecithin and choline on blood serum vitamin A following the feeding of vitamin A (5 μg daily) or carotene (20 μg daily) for 21 days

SUPPLEMENT IN ADDITION TO VITAMIN A OR CAROTENE	SERUM VITAMIN A (μG/100 ML)					
	Vitamin A supplemented groups (8 rats in each group)			Carotene supplemented groups (6 rats in each group)		
	Depletion diets			Depletion diets		
	(c)	(d)	Av.	(c)	(d)	Av.
Lecithin	32.9	37.4	35.1	42.5	37.3	39.9
Choline	...	...	...	45.2	42.3	43.7
None	36.5	41.5	39.0	45.2	39.9	42.5

results of the carotene supplementation are summarized in tables 6 and 7. When either lecithin or choline was fed with the carotene, the weight gains were greater (1% level of significance) than when carotene was fed with the basal diet alone. There was no significant carry-over effect of the depletion diets. The lecithin-supplemented group stored more

vitamin A (significant at the 1% level) than either of the other 2 groups, and choline produced no effect in this respect.

It will be noted that small amounts of pigment estimated as "carotene" were found in the livers following carotene feeding. Both "carotene" and vitamin A were converted to International Units (micrograms carotene × 1.66 plus micrograms vitamin A × 3.33) and the percentage storage calculated. While the general pattern of storage on the various diets was similar to that when vitamin A was fed (experiments I and II), a higher percentage storage was obtained when 20 μg of carotene were fed.

TABLE 8

Influence of sex on the liver storage of vitamin A in rats fed 5 μg of vitamin A or 20 μg of carotene daily

SUPPLEMENT IN ADDITION TO VITAMIN A OR CAROTENE	NO. OF RATS	AVERAGE LIVER STORAGE (μG)		DIFFERENCE BETWEEN ♂ AND ♀ OF SAME GROUP	DIFFERENCE BETWEEN ♂ OF LECITHIN AND NON-LECITHIN GROUP	DIFFERENCE BETWEEN ♀ OF LECITHIN AND NON-LECITHIN GROUP
		♂	♀			
				%	%	%
Vitamin A-fed groups						
None	16	14.6	20.7	41.6 [1]	...	...
Lecithin	16	19.4	24.3	25.1 [2]	32.5 [1]	17.1 [3]
Carotene-fed groups						
None	12	45.4	65.3	43.8 [1]	...	...
Choline	12	44.6	66.8	49.8 [1]	...	...
Lecithin	12	56.8	76.5	34.7 [1]	25.1 [2]	17.2 [3]

[1] Significant at the 1% level (probability of happening by chance 1 in 100).
[2] Significant at the 5% level (probability of happening by chance 1 in 20).
[3] Not significant.

As in the previous experiments, a sex difference in vitamin A storage was evident. The liver storage data are summarized by sex in table 8. The sex difference in liver vitamin A storage was highly significant, regardless of the supplement fed in addition to carotene.

Again, as in the previous experiments, the blood plasma vitamin A was lower in the group which received both lecithin and carotene.

DISCUSSION

Several investigators (Brenner et al., '42; Davies and Moore, '35; Horton et al., '41; Popper and Greenberg, '41) have observed the rapid loss of vitamin A reserves in rats fed a vitamin A-free diet. The feeding of lecithin appears to hasten the decline in liver stores, but the superior growth response indicates either that the loss of the vitamin by destructive breakdown is retarded, or that greater economy is effected in its functional metabolic processes.

Adlersberg and Sobotka ('43b) and Augur et al ('47) have shown that lecithin enhances the digestion and absorption of fat, and Muelder and Kelly ('42) have produced evidence that a minimum dietary fat level is necessary for the optimum absorption and utilization of vitamin A, which in turn is responsible for greater weight gains. In the experiments reported herein no vitamin A was fed during the depletion period, and the enhanced growth rate accompanied by lower levels of vitamin A in both the blood and liver which resulted from the addition of lecithin to the depletion diet indicates a primary effect on the mobilization and utilization of the vitamin A reserves.

The greater growth response and liver storage of vitamin A in the rats receiving both lecithin and vitamin A indicate clearly both increased absorption and more effective utilization. The slight carry-over effect of lecithin feeding during the depletion period in increasing growth and liver storage during the vitamin A repletion period also supports this view.

Greenberg and Popper ('41) and Brenner et al. ('42) demonstrated the presence of sufficient vitamin A in the retina and blood even in the absence of liver stores. This probably explains the continuation of growth toward the end of the depletion period and indicates that lecithin may have aided in the mobilization and effective utilization of the vitamin from body depots other than the liver. The fact that lower blood levels of vitamin A were observed in the animals being fed lecithin, even though they gained more weight and stored

more of the vitamin in the liver, is further evidence that lecithin aids in vitamin A utilization.

The results of these experiments support the observations that lecithin feeding increases liver vitamin A storage, as reported by Slanetz and Scharf ('45), and conserves the hepatic reserves of this vitamin, as reported by Polskin ('40).

Crude soya lecithin contains a number of compounds that might be suspected of being responsible for the observations on growth and liver storage of vitamin A. Scharf and Slanetz ('44) and Patrick and Morgan ('44) presented evidence that α-tocopherol was not the active principle responsible for these effects. Since choline plays an important role in fat metabolism, we considered it of interest to compare its effects with those of lecithin under similar experimental conditions.

Choline feeding did not result in greater weight gains during the depletion period or in greater weight gains or increased liver storage of the vitamin during the vitamin A repletion period. The significant carry-over effect of choline feeding during the depletion period in decreasing the growth response to vitamin A feeding is difficult to explain. Thorbjarnarson and Drummond ('38) observed that vitamin A depletion could be accelerated by causing a partial dispersal of fat from the liver by the administration of choline. This effect was not evident from our experiments. Our choline-fed rats actually grew as well as the control group during the depletion period and they did not show deficiency symptoms at an earlier date.

It is possible that the lecithin effect is due to the fatty acids of lecithin, since it has been shown by Sherman ('40, '42) that some unsaturated fatty acids aid in the absorption and metabolism of vitamin A. A more probable explanation is that the lecithin molecule acts as an intact unit, or that the crude lecthin used contains some compound other than lecithin, choline or α-tocopherol which is the active principle.

The greater liver storage of vitamin A observed when carotene was fed in these experiments suggests the possibility

that, up to a certain point, as the level of intake is increased, the percentage stored is increased.

The evidence in the literature regarding sex differences in ability to store vitamin A is conflicting. Lemley et al. ('47) reported that there is no sex difference. The results of our study support the observation of Brenner et al. ('42) that under comparable experimental conditions females store appreciably more vitamin A in the liver than males. The addition of lecithin to the ration enables either sex to store greater amounts of vitamin A in the liver when either 5 μg of vitamin A ester or 20 μg of carotene are fed daily.

SUMMARY

A series of 3 experiments were undertaken to study the effects of crude soya lecithin and choline on the absorption, utilization and storage of vitamin A in laboratory rats. The following pertinent observations were made.

When 2% of the basal vitamin A-free diet consisted of lecithin (⅕ or ⅙ of the fat replaced by lecithin), greater weight gains during the depletion period were obtained than when 0.07% choline or a similar diet without added lecithin was fed. Limited data indicate that liver reserves of vitamin A were exhausted slightly more rapidly when the depletion diet contained added lecithin.

The feeding of lecithin during the depletion period had a slight, although not a statistically significant, carry-over effect in increasing the weight gains during the vitamin A repletion period. On the other hand, choline feeding during depletion resulted in significantly smaller weight gains during the following vitamin A repletion period.

The feeding of lecithin with a vitamin A or carotene supplement resulted in greater gains in weight and increased liver storage of vitamin A. When choline was fed with these supplements, the liver storage of vitamin A was no different from that of the groups which received the vitamin supplement alone.

At the levels fed, there was a greater percentage storage of vitamin A when β-carotene was the supplement, although the weight gains of the vitamin A-supplemented groups were greater.

Females consistently stored more of the vitamin in the liver under all the dietary conditions studied.

The data indicate that lecithin enhances both the absorption and utilization of vitamin A and carotene. The choline fraction of the lecithin molecule is not responsible for this effect.

ACKNOWLEDGMENT

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DISTRIBUTION AND FRACTIONATION OF THE MONKEY ANTI-ANEMIA FACTOR[1]

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ONE FIGURE

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Extensive studies in our laboratory on the nutritional requirements of the rhesus monkey (Macaca mulatta) have shown that this species will grow at a normal rate for extended periods of time when given a purified ration supplemented with all of the known crystalline B vitamins. However, when individual vitamin deficiencies were studied, it was found that the monkey frequently would fail to respond completely to the administration of the missing vitamin. This was particularly evident in the case of riboflavin, pyridoxine, pantothenic acid and folic acid deficiencies (Cooperman et al., '45; McCall et al., '46; Cooperman et al., '46a). The inclusion of unenriched corn grits in the basal ration at a level of 40% also precipitated the condition (Cooperman et al., '46b). The anemia as well as the lack of growth resulting from these deficiencies was counteracted by certain crude materials such as liver and milk. Consequently, it was postulated that a new factor was involved, and it was tentatively named the monkey anti-anemia factor (Cooperman et al., '45). The assay procedure developed for measuring this factor consisted of omit-

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ting a vitamin such as riboflavin from the regimen until the monkey had lost weight and developed an anemia. At this time, vitamin therapy was instituted with the result that the monkey would respond suboptimally to the vitamin. If a preparation fed at this point would bring about a weight and hematological response, it was concluded that it contained the new factor. In the case of the corn grit ration, the condition was precipitated even in the presence of all B vitamins. Administering active preparations brought about a weight and hematological response comparable to that observed on the vitamin deficient rations.

We are reporting in this paper studies on potential sources of the anti-anemia factor, fractionation procedures and possible methods for improving the assay procedure.

EXPERIMENTAL

The housing, care and handling of the monkeys have been described previously (Waisman et al., '43). The basal ration had a percentage composition of sucrose 73, purified casein 18, salts IV 4, corn oil 2 and cod liver oil 3, with 40% of the "dry mix" replaced by corn grits (Cooperman et al., '46b). Adequate amounts of thiamine (1 mg), riboflavin (1 mg), pyridoxine hydrochloride (1 mg), niacin (5 mg), pantothenic acid (3 mg), choline chloride (25 mg), i-inositol (50 mg), p-aminobenzoic acid (50 mg), biotin (0.02 mg), folic acid (0.1 mg), and ascorbic acid (50 mg) were fed daily as a separate supplement.

Monkeys placed on the corn grit ration grew normally for 3–10 months before showing deficiency symptoms. Eventually a plateau or loss in weight occurred, an anemia developed and the animals died unless further supplementation was given. In the assay procedure, it was required that all animals show a plateau or weight loss for at least 1 month before administration of the test material. A 1-month interval was generally allowed in order to ascertain the activity of the sample as evidenced by a weight response and increase in hemoglobin concentration of the blood. Various substances were assayed using this technique, and it was found that fresh liver or whole

dry liver substance as well as malt sprouts and milk constituted the best sources of the factor (Ruegamer et al., '47a). Furthermore, upon preliminary fractionation of milk, it was found that the activity occurred in the whey fraction (Cooperman et al., '46c).

On the basis of this work, a sample of whey solids [2] was fed to 1 monkey at a level of 3% of the basal ration. The initial weight of the animal was 5020 gm and the hemoglobin 12.6 gm %. Since no response was seen after the first week, the level of whey solids was increased to 6%. No response was observed at the end of the third week, and the preparation was discontinued (table 1). An active liver preparation was then fed for 1 month and the weight of the animal increased to 5400 gm, and the hemoglobin rose to 13.7 gm %.

Since our regular monkeys were frequently kept on a ration consisting mainly of raw potatoes and carrots and seemed to remain in a fair state of health, 50 gm of raw potatoes and carrots were fed to 2 deficient monkeys, respectively. The raw potato failed to produce a response, whereas the addition of a liver preparation did produce a gain of 540 gm in 2 months. The carrot supplementation likewise failed to produce any increase in either body weight or hemoglobin (table 1).

In the microbiological assay with Streptococcus faecalis (American type culture collection no. 8043), whole yellow corn was found to give a greater growth stimulation than corn grits (Ruegamer et al., '47a; Cooperman et al., '46d). The activity measured by the *S. faecalis* assay was originally thought to parallel that of the monkey anti-anemia factor. Consequently 20% whole yellow corn was fed to 1 monkey at the expense of the corn grits in the basal ration. No activity could be seen as evidenced by the failure to produce either a growth or hemoglobin response in the monkey (table 1). Likewise, yeast extract [3] which was active for the microörganism, was fed to 1 monkey at levels as high as 6% without

[2] Western Condensing Company, Appleton, Wis.
[3] Difco.

TABLE 1

Data showing growth and hemoglobin responses to various preparations tested for the monkey anti-anemia factor.

EXPT. NO.	MONKEY NO.	PREPARATION	DURATION OF TEST	INITIAL WT.	FINAL WT.	INITIAL Hb.	FINAL Hb.
				gm	*gm*	*gm %*	*gm %*
1	249	6% whey solids	3 wks.	5020	5100	12.64	12.33
	340	Acetone extr. ≅ 5 gm W.L.S.[1]	1 mo.	3580	3920	12.91	14.0
2	295	50 gm raw potato/day	3 wks.	4600	4650	11.63	10.43
	295	Norite eluate ≅ 18 gm fresh liver/day	2 mo.	4700	5240	8.82	12.76
3	297	50 gm raw carrot/day	3 wks.	4640	4680	10.6	10.01
4	189	20% whole yellow corn	1 mo.	5400	5460	13.3	13.33
5	296	6% Difco yeast extr.	1 mo.	3880	3940	12.33	12.14
	296	Alc. extr. liver ≅ 9 gm/day	1 mo.	3930	4380	11.93	13.89
6	341 *	0.5% L-lysine plus 0.5% DL-tryptophane	1 mo.	3850	3900	12.91	13.17
7	277	0.5 mg folic acid/day	3 wks.	4220	4230	13.38	12.06
8	248	50 mg rutin + 50 mg hesperitin per day	1 mo.	5060	5120	....	....
9	276	1 U.S.P. unit/day of reticulogen injected	3 wks.	3260	3300	13.38	13.23
	277	15 units/day injected	3 wks.	3300	3300	13.03	13.03
	276	45 units/day injected + 0.25 mg B_c conjugate	2 wks.	3500	3570	13.30	13.01
	277	45 units/day orally	3 wks.	3280	3280	12.56	10.89
	276	15 units/day + 24% casein	1 mo.	4200	4200	12.06	12.14
	277	15 units/day orally + B_c conj. (0.25 mg)	2 wks.	3300	3280	13.03	12.56
10	276	1 gm fresh liver/day	2 wks.	5000	4950	14.28	14.28
	276	3 gm fresh liver/day	3 wks.	4950	5050	14.28	14.55
	276	4 gm fresh liver/day	2 wks.	5050	5200	14.55	14.55
	297	4 gm fresh liver/day	3 wks.	3830	4300	10.43	13.03
11	249	60% alc. extra. ≅ 4 gm liver/day	3 wks.	4960	5000	13.03	12.64
	368	Alc. extr. ≅ 6 gm liver/day	2 wks.	3240	3300	12.76	12.76
	368	≅ 9 gm liver/day	1 mo.	3300	3680	12.76	13.89
	277	≅ 9 gm liver/day	5 wks.	3280	3820	10.89	14.08
	296	≅ 9 gm liver/day	1 mo.	3930	4380	11.93	13.89
	340	≅ 9 gm liver/day	3 wks.	4680	5000	13.38	14.97
12	276	60% Alc. extr. malt sprouts ≅ 10 gm/day	1 wk.	3620	3620	13.30	....
	276	≅ 30 gm/day	2 wk.	3620	3650	13.30	12.63
	276	≅ 50 gm/day	1 mo.	3650	3820	13.03	13.4
13	277	Alc. extr. tomatoes ≅ 20 gm/day	2 wks.	4220	4250	14.08	....
	277	≅ 40 gm/day	1 wk.	4250	4200	14.08	13.38
	297	≅ 60 gm/day	2 wks.	4700	4750	12.06	10.09
14	340	Acetone extr. ≅ 5 gm W.L.S./day	1 mo.	3580	3920	12.5	16.1
15	297	Norite eluate ≅ 12 gm liver/day	3 wks.	4430	4430	12.56	10.66
	297	≅ 18 gm/day	3 wks.	4430	4640	10.66	11.21
	295	≅ 18 gm/day	1 mo.	4900	5200	8.82	12.76

[1] Whole liver extract.

any beneficial effect. An active liver preparation fed after the yeast was removed from the ration brought about a weight response of 450 gm and a hemoglobin response from 11.93 gm to 13.89 gm % in 1 month (table 1 and fig. 1, monkey 296).

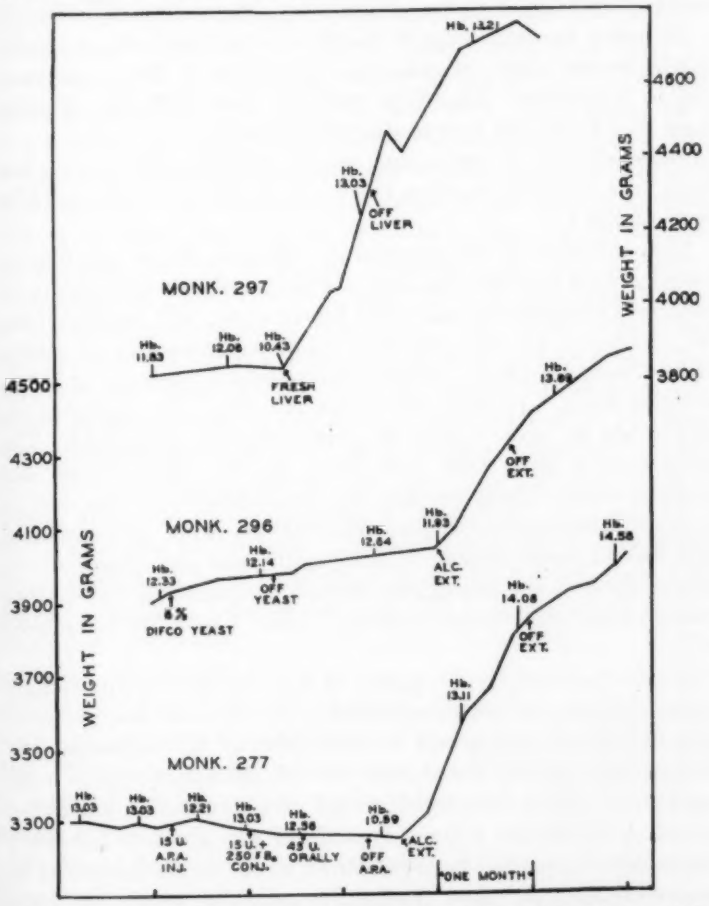

Fig. 1 Typical growth curves and hemoglobin values for 3 monkeys receiving liver preparations. The alcohol extract indicated on the curves was prepared from fresh liver and fed by stomach tube at a level ≅ 9 gm per day. The fresh liver homogenate given monkey 297 was fed at a level of 4 gm per day.

Since the inclusion of 40% corn grits lowered the lysine and tryptophane contents of the casein ration, 0.5% L-lysine and 0.5% DL-tryptophane were fed as supplements mixed in the basal ration. However, no response could be seen in a deficient monkey and this supplementation was discontinued (table 1).

In order to determine if the folic acid level (0.1 mg/day) might be low, daily supplements of 0.5 mg of folic acid were fed to 1 monkey. No effect could be seen and the original level of 0.1 mg/day was continued (table 1).

When 50 mg of rutin and 50 mg of hesperidin were fed daily to a deficient monkey for 1 month, no response could be seen (table 1).

Concentrates of the pernicious anemia factor have been found active for the dog, rat, chicken and microörganism *S. faecalis* (Ruegamer et al., '47b; Sporn et al., '47; Betheil et al., '47; Nichol et al., '47; unpublished data). Samples of reticulogen [4] were injected intramuscularly into 2 monkeys at levels ranging from 1 to 45 U.S.P. units per day. No apparent effect could be seen either in hemoglobin or in weight. The concentrates were then fed orally at the same levels, but without effect. Thinking that the factor might in some way be a protein utilization factor, the extracts were given with a 24% casein basal ration. B_c conjugate was also given simultaneously with the pernicious anemia preparation, but neither combination showed any activity (table 1 and fig. 1, monkey 277).

On the basis of these results, it was decided to fractionate some of the more active materials. In all cases the preparations were fed by stomach tube because of the unpalatability of some of them for the monkey. First, the approximate minimal level of fresh liver which would give a response was determined. Accordingly, 1 gm of fresh liver was given to 1 monkey daily. No effect could be seen after 2 weeks so the level was increased to 3 gm per day. A slight response was seen, but when the level was raised to 4 gm, a good response of 150 gm gain in body weight followed. A liver homogenate was given to an-

[4] Eli Lilly and Company, Indianapolis, Ind.

other monkey at a level of 4 gm per day, and this animal showed a response of 470 gm in weight and 2 gm % in hemoglobin in 3 weeks (fig. 1, monkey 297). Therefore, it was concluded that between 3 and 4 gm of fresh liver is the minimal amount necessary for a good response.

An ethanol extract was made from fresh beef liver by grinding the liver in a meat grinder and adding 95% ethanol until the ethanol concentration was 60%. The suspension was stirred for 1 hour and allowed to stand overnight. The extract was then removed and the residue re-extracted with 60% ethanol. The extracts were combined and filtered through filter cell. After removing the ethanol by vacuum distillation, the preparation was extracted with ether and the ether layer was discarded. The aqueous phase was concentrated further to a 10:1 concentration and stored at 0°C. This extract was fed by stomach tube at a level equivalent to 4 gm of fresh liver per day. No effect could be seen in 2 weeks so the level was raised to the equivalent of 6 gm of fresh liver. Still no effect was observed and the level was further increased to the equivalent of 9 gm of fresh liver per day. At this level, a weight response of 380 gm and a hemoglobin response of 1 gm % was seen in 1 month. Three other monkeys were given the extract at a level equivalent to 9 gm of fresh liver and similar responses were obtained (table 1). Therefore it was concluded that the ethanol extract was active at a level representing 9 gm of fresh liver per day. Furthermore, dry weight analysis showed that when 4 gm of fresh liver homogenate were fed, between 0.8 and 1 gm of solids was furnished per day. When the alcohol extract was fed at a level equivalent to 9 gm of fresh liver, only 270–300 mg of solids were furnished per day.

Methanol extracts of fresh beef liver prepared in the same manner were also active. However, when ethanol extracts were prepared from malt sprouts and fresh tomatoes, only borderline activity could be observed even when levels up to that equivalent to 50 gm of the original material were fed per

day. Aqueous extracts had been found to be quite active at lower levels (table 1).

An acetone extract was prepared by extracting whole liver substance [5] in a Soxhlet extractor for 24 hours. When this preparation was fed at a level the equivalent of 5 gm per day, the weight of the test animal increased 440 gm in 1 month and the hemoglobin increased from 12.91 to 14 gm % as seen in table 1.

On the basis of studies carried out with the rat (Jaffé and Elvehjem, '47), a norite eluate fraction from fresh liver was prepared and fed as a possible source of the monkey factor. The preparation was made from the methanol extract previously described by making the extract acid with concentrated HCl to methyl orange (red) and filtering. Norite [6] was added (20 gm/kg liver) and the solution was stirred for 1 hour. The solution was filtered in a Buchner funnel using a pad of filter cell. The adsorption procedure was repeated and the norite was washed with 50% methanol while still on the Buchner, the filtrate being discarded. Without suction, 150 ml of 1% phenol solution were added to the Buchner containing the norite, and the solution was allowed to drain for ½ hour. After stirring carefully, suction was applied and the filtrate collected. Approximately 8 elutions were carried out in this manner and the eluates were combined and extracted with ether until free from phenol. The eluate was then distilled under vacuum until a concentration equivalent to 60 gm of fresh liver per milliliter was obtained. When the eluate was fed to 2 monkeys at levels as high as that equivalent to 12 gm of fresh liver per day, no activity could be observed. If the level was increased to one equivalent to 18 gm of fresh liver, good responses in weight and hemoglobin were obtained in both monkeys (table 1). Dry weight analysis revealed that approximately 90 mg of solids were being fed per day as compared to 1 gm solids when fresh liver was used and 270–300 mg solids when the ethanol extract was used.

[5] Wilson and Company, Chicago, Ill.

[6] Darco G-60.

Since between 3 and 10 months are generally required to precipitate the deficiency in the monkey, several attempts were made to develop a more rapid means of estimating the activity of various preparations. A microbiological method using *S. faecalis* was tried in the hope that the activity observed with *S. faecalis* would parallel that seen with the monkey. As the study progressed, however, it was found that the microörganism and the monkey were not responding to the same factor. Therefore the microbiological assay was discontinued and alterations in the diet of the monkey were tried. Since better results had been obtained in the rat assay on a natural ration as compared to a purified diet (Sporn et al., '47), a 50-50 corn-soybean ration with added salts (2%), vitamins and cystine was fed to 2 monkeys. These animals found the ration unpalatable; therefore, the experiment was discontinued.

Because of early successes in showing deficiencies in rats by feeding sulfa drug, 1% sulfasuxidine was included in the ration of 1 monkey for a period of 2½ months. During this time the monkey gained 350 gm and showed no depression in the hemoglobin level.

The protein level was reduced in the case of another monkey to 12% casein. This animal continued to grow approximately 300 gm over a 3-month period so the regimen was discontinued.

Because of results obtained in dog studies involving the relationship of folic acid to fat metabolism, 4 monkeys were fed a fat free ration in which the fat of the basal purified diet was replaced by sucrose. Two of these monkeys, furthermore, received no folic acid whereas the other 2 received adequate amounts of folic acid. After 2 months on this regimen, the monkeys were still growing slowly, and no significant blood changes occurred.

None of the proposed procedures for shortening the assay has been found to be successful, but this phase of the work is being continued.

DISCUSSION

When a sample of commercial whey solids was fed at a level of 6%, no activity could be obtained for the monkey anti-anemia factor. Apparently the factor was destroyed in the processing of the whey since fresh whey was found to possess full activity.

Fresh samples of raw carrots, raw potatoes, corn and soybeans were fed as sources of the monkey factor, but all were found to be inactive even when fed at levels 10 times that of liver. Liver still remained the best starting material for fractionation work.

High levels of folic acid and pernicious anemia preparations also failed to show activity. From these data it may be concluded that the monkey factor is separate from the rat, chicken and dog factors since all 3 of these species responded to pernicious anemia concentrates.

When liver extracts were employed, it became necessary to give the preparations by stomach tube as the monkeys found them unpalatable. This technique of administering the liver preparations in single doses may be open to criticism in view of newer theories which propose that for optimum growth, all essential nutrients must be present within the animal at the same time. However, since good responses were observed using this procedure, we believe it to be a satisfactory method for giving the preparations.

Approximately 4 gm of fresh liver are needed per day when given by stomach tube. When a 60% methanol or ethanol extract of fresh liver was prepared and fed to monkeys, good responses could be obtained when equivalents of 8 or 9 gm of fresh liver were fed per day. This would mean that approximately ½ of the activity was lost by this step, but the solids decreased from 800–1000 mg found in 4 gm of fresh liver to 270–300 mg contained in the alcohol extract when fed at a level equivalent to 9 gm per day. Thus a threefold concentration was obtained by extracting the fresh liver with alcohol. In the case of the norite eluate, the equivalent of 18 gm of fresh liver was needed. However, dry weight analysis re-

vealed that the solids furnished by this equivalent amount of fresh liver amounted to only 90 mg per day. Therefore, an overall approximate tenfold concentration was realized by the norite elution procedure.

SUMMARY

Further studies were carried out on the distribution and fractionation of the monkey anti-anemia factor. Whey solids, yellow corn, raw potato and raw carrot are poor sources of the factor. Higher levels of folic acid and rather high levels of rutin and hesperidin, L-lysine and DL-tryptophane, and pernicious anemia concentrates failed to show any activity for the monkey.

Approximately 4 gm of fresh liver are needed by the monkey per day. An ethanol extract of fresh liver was active as well as a methanol and acetone extract of liver. A norite eluate prepared from the alcohol extract also proved active when fed at a higher level. A tenfold concentration was observed by the norite elution method.

Atttempts were made to shorten the assay by feeding sulfasuxidine in the ration, lowering the protein intake, and by feeding fat free rations.

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FURTHER STUDIES ON THE RELATIONSHIP OF NICOTINIC ACID, TRYPTOPHANE AND PROTEIN IN THE NUTRITION OF THE PIG[1]

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The requirement of the pig for nicotinic acid has been observed by many workers; however, Wintrobe and coworkers ('45) reported that nicotinic acid deficiency could not be produced in young pigs fed a purified ration containing 26% casein. In a paper previous to the present one Luecke et al. ('47) reported that nicotinic acid deficiency can be produced in young pigs fed a low protein ration composed largely of corn, and that supplements of tryptophane were effective in preventing this deficiency. It was noted, however, that daily supplements of 200 mg of DL-tryptophane per pig were not completely effective in preventing the characteristic intestinal lesions of nicotinic acid deficiency. With this in mind, it seemed worthwhile to determine whether higher levels of tryptophane would be effective in completely preventing nicotinic acid deficiency. Furthermore, since Powick and coworkers ('47) were not able to confirm the work of Wintrobe et al. ('45) regarding the production of nicotinic acid deficiency using rations containing high levels of casein, it was

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decided to determine whether the deficiency symptoms could be produced in a high protein ration made up largely of corn.

EXPERIMENTAL

Pigs used in this experiment were farrowed by Duroc Jersey sows which had been bred to a Yorkshire boar. Two weeks before the pigs were placed on the experiment they were vaccinated for hog cholera, using the serum and virus method. The 3 basal rations used are shown in table 1. The crude protein content of rations A, B, and C was 15.1, 19.4 and 29.6% respectively, and the nicotinic acid content was 11.2, 8.7 and

TABLE 1

Composition of rations [1]

	RATION		
	A	B	C
	%	%	%
Corn	87	80	68
Casein (commercial)	5.5	12	25
Soybean oil meal	5.5	6	5
Complex mineral mixture	2	2	2

[1] The following amounts of vitamins were supplied daily to each animal: thiamine, 10 mg; riboflavin, 10 mg; calcium pantothenate, 25 mg; and pyridoxine, 6 mg.

8.1 mg per pound respectively, as determined microbiologically by the method of Krehl, Strong and Elvehjem ('43). It can be seen that the nicotinic acid content of the 3 rations decreased as the level of casein increased, due to the fact that commercial casein is extremely low in nicotinic acid. This fact serves a very useful purpose since, even though the higher levels of casein stimulate greater feed consumption, the actual intakes of nicotinic acid are quite uniform.

The tryptophane content of rations A, B, and C was 0.13, 0.20 and 0.41% respectively, as determined microbiologically using the organism *Lactobacillus arabinosus*. The medium used for the tryptophane assay was essentially the same as

that described by Sauberlich and Baumann ('46), and the samples were prepared for assay according to the method of Wooley and Sebrell ('45). The 3 rations were supplemented with vitamins A and D in amounts which supplied 2,000 I.U. of vitamin A and 200 I.U. of vitamin D per pound of feed. Thiamine, riboflavin, calcium pantothenate and pyridoxine were fed daily to each pig in the amounts indicated (table 1). The composition of the complex mineral mixture was the same as that described previously (Luecke et al., '47).

At the conclusion of the 6-week experimental period the animals were autopsied and pathological studies made of the affected tissues.

RESULTS

Pigs in lot 1 were fed the low protein ration A plus daily supplements of 30 mg of nicotinic acid. The growth response of these pigs is shown in table 2. The animals in this lot

TABLE 2

Response of pigs fed the various experimental rations (Five pigs in each lot on trials lasting 6 weeks)

	LOT				
	1	2	3	4	5
Ration fed	A[1]	A	A[2]	B	C
Initial age in weeks	5	5	5	6	5
Average initial weight (lbs.)	22	22	22	27	22
Average daily gain (lbs.)	0.61	0.26	0.84	0.74	1.05
Average daily feed consumption (lbs.)	1.37	1.05	1.75	2.00	2.06
Lbs. of feed per lb. of gain	2.25	4.04	2.08	2.70	1.96

[1] Pigs in lot 1 received daily supplements of 30 mg of nicotinic acid.
[2] Pigs in lot 3 received daily supplements of 1 gm of DL-tryptophane.

gained an average of 0.61 pounds per day while consuming 1.37 pounds of feed. The growth response of these pigs was rather surprising, inasmuch as the protein content of ration A was lower than is generally recommended for pigs of that age and weight. The pigs in this lot required 2.25 pounds of feed to produce 1 pound gain in body weight.

Pigs in lot 2 were fed ration A. During the 4th week typical symptoms of nicotinic acid deficiency were noted. These included diarrhea, a rough haircoat and lowered appetite. The intestinal lesions of nicotinic acid deficiency have been previously described (Luecke et al., '47). The pigs in this lot required 4.04 pounds of feed to produce 1 pound of gain.

Ration A plus daily supplements of 1 gm of DL-tryptophane were fed to the pigs in lot 3. In a previous experiment (Luecke et al., '47) daily supplements of 200 mg of DL-tryptophane produced good growth, but did not entirely prevent the typical intestinal lesions of nicotinic acid deficiency. Inasmuch as it seemed probable that the 200 mg level was too low, the supplementary level of tryptophane was increased to 1 gm. The growth rate of the pigs in this lot was excellent (table 2). These pigs gained an average of 0.84 pounds per day while consuming 1.75 pounds of feed, and it required only 2.08 pounds of feed to produce 1 pound of gain. No intestinal lesions characteristic of nicotinic acid deficiency were found in the animals of this lot upon subsequent autopsy. This indicates that adequate levels of tryptophane will completely prevent symptoms of nicotinic acid deficiency in the pig.

Pigs in lot 4 were fed ration B and gained an average of 0.74 pounds per day (table 2), which is somewhat better than the daily gains produced by the animals in lot 1. However, the pigs in lot 4 required 2.70 pounds of feed to produce 1 pound gain in body weight as against 2.25 pounds for the animals in lot 1. During the 5th week of the experiment 2 of the pigs in lot 4 developed diarrhea, and upon autopsy at the conclusion of the experiment 3 animals showed evidence of enteritis; the remaining 2 pigs were normal. The intestinal lesions found at autopsy in the 3 pigs of this lot were not as severe as those found in the animals of lot 2 fed low protein ration A. However, it seemed evident that even though the crude protein content of ration B was high, it did not contain sufficient tryptophane to counteract completely the low nicotinic acid content.

Pigs in lot 5 were fed ration C. The growth response of these animals was excellent (table 2). They gained weight more rapidly and efficiently than any other experimental lot, requiring only 1.96 pounds of feed to produce 1 pound gain in body weight. No evidence of nicotinic acid deficiency was noted, either during the experiment or upon subsequent autopsy.

Luecke and coworkers ('47) have shown that one of the symptoms of nicotinic acid deficiency in the pig is a low urinary excretion of N^1-methylnicotinamide. Accordingly, during the 6th week of the experiment the pigs were placed

TABLE 3

Average daily intakes of tryptophane and nicotinic acid and excretion of N^1-methylnicotinamide

LOT	TRYPTOPHANE		NICOTINIC ACID		N^1-METHYL-NICOTINAMIDE
	In feed	Supplement	In feed	Supplement	
	gm	*gm*	*mg*	*mg*	*mg*
1	0.80		15.34	30	13.87
2	0.61		11.76		4.62
3	1.12	0.50 [1]	19.60		18.14
4	1.80		17.40		9.83
5	3.80		16.70		15.06

[1] Assuming D-tryptophane to be completely inactive.

in metabolism cages and a 24-hour collection of urine obtained. The average daily excretions of N^1-methylnicotinamide, as determined by the method of Huff, Perlzweig and Tilden ('45) are shown in table 3. It should be noted that the average excretion of N^1-methylnicotinamide for the pigs in lot 2 was considerably lower than that of lot 4. This agreed with the results obtained at autopsy in that the intestinal lesions found in pigs of lot 2 were far more severe than those found in lot 4.

Since the nicotinic acid and tryptophane contents of rations A, B and C were known, the average daily intakes of these substances could be calculated from the average daily

feed consumption. The results of these calculations are shown in table 3. It seems evident that the addition of nicotinic acid to the low protein ration A increased the utilization of dietary tryptophane, since the animals in lot 1 grew normally on an average daily intake of 0.80 gm of tryptophane, while in the case of lot 4 the daily ingestion of 1.80 gm gave only a slightly better growth rate and, as a matter of fact, resulted in a lower efficiency of feed utilization. The fact that the supplementation of ration A with DL-tryptophane resulted in good growth indicates that tryptophane is the limiting amino acid in this ration. On the basis of tryptophane intake it is rather surprising that the pigs in lot 3 grew more rapidly and efficiently than the animals in lot 4. If it is assumed that D-tryptophane is inactive for the pig, then the animals in lot 3 were actually consuming less of this amino acid than the pigs in lot 4. Although the present experiment offers no proof, it seems possible that the pig may be able to utilize D-tryptophane to some extent. Another possibility is that the addition of synthetic tryptophane stimulates nicotinic acid synthesis which, in turn, improves tryptophane utilization. This possibility has already been suggested by Krehl et al. ('46).

DISCUSSION

In the experimental production of nicotinic acid deficiency in pigs, the age and weight of the pig are of great importance. It has been the authors' experience that symptoms of nicotinic acid deficiency are produced more readily in pigs weighing less than 30 pounds. Furthermore, we have never been able to produce nicotinic acid deficiency in pigs weighing 60 pounds or more when fed a low protein ration made up largely of corn. Braude, Kon and White ('46) were also unable to produce nicotinic acid deficiency in older pigs.

The fact that nicotinic deficiency could not be produced on a corn ration containing 25% casein confirms the work of Wintrobe et al. ('45). Although the pigs receiving the high protein ration were ingesting 16.7 mg of nicotinic acid per day (table 3), it is not likely that this amount of nicotinic acid

would prevent deficiency, since in the case of lot 4 the pigs ingested 17.4 mg per day and showed mild deficiency symptoms.

Since the nicotinic acid requirement of the pig is directly related to the tryptophane intake, the difficulties encountered in attempting to elucidate the requirement for this vitamin are obvious. The use of purified rations in estimating the nicotinic acid requirement of the pig (Hughes, '43) will probably give results lower than those obtained by using a ration made up largely of corn.

SUMMARY

Adequate amounts of DL-tryptophane as supplements to a low protein corn ration will prevent nicotinic acid deficiency in the pig. Supplementation of the same low protein ration with nicotinic acid seems to increase the utilization of dietary tryptophane.

No symptoms of nicotinic acid deficiency were produced on a corn ration containing 25% casein. Moreover, pigs fed this high protein ration grew very rapidly and required only 1.96 pounds of feed to produce 1 pound gain in body weight.

Mild symptoms of nicotinic acid deficiency were produced in pigs whose calculated average daily intake of nicotinic acid was 17.4 mg.

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